

STATEMENT

ACTIN MYOSIN AND THE

MOVEMENTS OF

NON-MUSCLE CELLS

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of

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by

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## STATEMENT

Collaboration was obtained from Dr. M.J. Stewart in connection with the experiments described in Chapter 7. Dr. Stewart also wrote the computer programme for the statistical analysis described in Chapter 4. Assistance with gel electrophoresis was provided by Mr. D.O. Irving. In all other respects, the work reported in this thesis was performed by me and no material except where due reference is made has been submitted for publication elsewhere.

*Jakajir*  
7.12.78



# TABLE OF CONTENTS

	<u>Page</u>
ACKNOWLEDGEMENTS	vi
PUBLICATIONS AND RESEARCH COMMUNICATIONS	viii
ABSTRACT	x
 CHAPTER 1 GENERAL INTRODUCTION	 1
Types of Cell Movements	3
(a) <i>Cell Migration</i>	3
(b) <i>Subcellular motility</i>	4
(c) <i>Contraction of muscle cells</i>	5
Mechanisms of Cell Motility	6
The Molecular Basis of Skeletal Muscle Contraction	9
Properties of Non-muscle Actin	10
Properties of Non-muscle Myosin	13
Localization and Form Determination of Contractile Proteins in Non-muscle Cells	16
(a) <i>Localization of actin filaments</i>	17
(b) <i>Localization of myosin</i>	18
Specific Aims of this Thesis	19
Technical Approaches	20
Experimental Model	21
 CHAPTER 2 MATERIALS AND METHODS	 23
Techniques related to Cultured Chick Embryo Connective Tissue Cells	26
(a) <i>Culturing of cells</i>	26
(b) <i>Examination of normal cells</i>	30
(c) <i>Glycerination</i>	30

(d) <i>Myosin extraction</i>	32
(e) <i>Heavy meromyosin (HMM) labelling of cells</i>	33
(f) <i>DNAase I treatment of cells</i>	35
Techniques related to Actin and Myosin Isolated from Rabbit Skeletal Muscle	36
(a) <i>Preparation of acetone-powder of actin</i>	36
(b) <i>Purification of actin from acetone-powder</i>	38
(c) <i>Isolation and purification of myosin</i>	39
(d) <i>Preparation of heavy meromyosin</i>	41
(e) <i>Model experiments with actin and myosin</i>	42
Techniques related to Chicken Gizzard Smooth Muscle	44
(a) <i>Isolation of intermediate filaments</i>	44
(b) <i>DNAase I treatment of intermediate filaments</i>	46
(c) <i>Trypsin treatment and HMM labelling of intermediate filaments</i>	46
Microscopical and Gel Electrophoretic Techniques	47
(a) <i>Light microscopy</i>	47
(b) <i>Electron microscopy</i>	48
(c) <i>Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis</i>	54
Addendum	56A
CHAPTER 3 STRUCTURE AND MOVEMENTS OF NORMAL CULTURED CHICK EMBRYO CONNECTIVE TISSUE CELLS: A LIGHT MICROSCOPIC, FINE STRUCTURAL AND BIOCHEMICAL STUDY	59
INTRODUCTION	60
RESULTS	63
Light (Phase Contrast) Microscopy of Living Cells	63

Electron Microscopy	65
(a) <i>General description of the fine structure of fixed CPD cells</i>	65
(b) <i>Description of the fine structure of filament systems of cells</i>	67
(c) <i>Histogram showing the diameter distribution of the various filaments</i>	70
SDS Polyacrylamide Gel Electrophoresis of Normal Cells	71
DISCUSSION	72
 CHAPTER 4    IDENTIFICATION AND LOCALIZATION OF F-ACTIN IN CYTOPLASMIC FILAMENTS: I STUDIES USING HEAVY MEROMYOSIN	 79
INTRODUCTION	80
RESULTS	83
HMM Labelling of Purified Muscle Actin Filaments	83
(a) <i>F-actin filaments</i>	83
(b) <i>Mg<sup>2+</sup> paracrystals of F-actin</i>	83
HMM Labelling of Glycerinated, Myosin-extracted Cells	84
(a) <i>Ultrastructure of cells without HMM</i>	84
(b) <i>Ultrastructure of cells with HMM</i>	85
HMM Labelling of Trypsin-treated, Glycerinated, Myosin-extracted Cells	86
(a) <i>Ultrastructure of cells without HMM</i>	86
(b) <i>Ultrastructure of cells with HMM</i>	87
DISCUSSION	88
 CHAPTER 5    IDENTIFICATION AND LOCALIZATION OF F-ACTIN IN CYTOPLASMIC FILAMENTS: II STUDIES USING DNAase I	 95
INTRODUCTION	96
RESULTS	99

(a) <i>Ultrastructure of negatively stained F-actin with and without DNAase I treatment</i>	99
(b) <i>Ultrastructure of <math>Mg^{2+}</math> paracrystals of actin with and without DNAase I treatment</i>	101
(c) <i>DNAase I action on sub-plasmalemmal filaments of glycerinated, myosin-extracted cells</i>	102
(d) <i>DNAase I action on cortical and sub-cortical (deeper) filaments</i>	103
(e) <i>SDS polyacrylamide gel electrophoretic analysis of the DNAase I extract</i>	106
DISCUSSION	106

CHAPTER 6 IDENTIFICATION, LOCALIZATION AND FORM DETERMINATION OF CYTOPLASMIC MYOSIN: STUDIES USING MYOSIN-EXTRACTING SOLUTIONS AND MUSCLE CONTRACTILE PROTEIN MODELS	114
INTRODUCTION	115
RESULTS	119
Ultrastructural Studies of Cells	119
(a) <i>Intact (unextracted) cells</i>	119
(b) <i>Glycerinated cells</i>	120
(c) <i>Cells extracted by 'one-step' myosin extraction</i>	121
(d) <i>Cells extracted by 'two-step' myosin extraction</i>	122
Ultrastructural Studies of Motility Proteins	123
(a) <i>Synthetic thick filaments of myosin from rabbit skeletal muscle</i>	123
(b) <i>Monomers of myosin</i>	124
(c) <i>F-actin and myosin monomer mixtures</i>	125
SDS Polyacrylamide Gel Electrophoresis of Extracts and Extracted Cells obtained after Glycerination and Myosin Extraction	125
DISCUSSION	127

CHAPTER 7	THE INTERMEDIATE FILAMENTS OF CHICKEN GIZZARD SMOOTH MUSCLE: A PRELIMINARY ULTRASTRUCTURAL AND BIOCHEMICAL ANALYSIS	135
	INTRODUCTION	136
	RESULTS	138
	(a) <i>Isolation of intermediate filaments</i>	138
	(b) <i>Ultrastructure of intermediate filaments</i>	139
	(c) <i>Effect of DNAase I on intermediate filaments</i>	139
	(d) <i>Effect of trypsin and HMM on intermediate filaments</i>	140
	(e) <i>Effect of aqueous extraction on the structure of intermediate filaments</i>	141
	DISCUSSION	141
CHAPTER 8	SUMMARY AND CONCLUSIONS	147
	Structures Associated with Moving Parts of the Cell	148
	F-actin Localization	149
	Myosin Localization	150
	Intermediate Filaments of Smooth Muscle	151
	Proposed Model	152
	Addendum	151A
	REFERENCES	154



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#### Publications

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With the aim of gaining a better understanding of the actomyosin-based aspects of the motility of non-muscle cells, attempts have been made to determine the form and localisation of actin and myosin in cultured chick embryo connective tissue cells.

Living cells were studied by phase contrast microscopy to gain an appreciation of the movements to be explained. Electron microscopic examination of fixed critical-point dried cells was carried out to determine the three dimensional distribution of the filamentous structures associated with the cells' moving components. Thin filaments (4-7 nm in diameter) were associated with movements of the cell surface and intermediate-sized filaments (7-11 nm) were associated with the movements of organelles. Both sets of filaments had attached to them tiny 'hook-like' structures with fine tails. These structures are frequently joined tail to tail to form 'dumb-bell' shaped structures.

Determination of the distribution of F-actin containing filaments was carried out with heavy meromyosin (HMM) and DNase I. After giving the glycerinated myosin-treated cells a gentle pre-treatment with trypsin, it was shown electron microscopically that virtually all filaments, both thin and intermediate labelled strongly with HMM, this indicating a content of F-actin. This conclusion was supported by the results of treating cells with DNase I (known to specifically depolymerize F-actin).

# ABSTRACT

With the aim of gaining a better understanding of the actomyosin-based aspects of the motility of non-muscle cells, attempts have been made to determine the form and localization of actin and myosin in cultured chick embryo connective tissue cells.

Living cells were studied by phase contrast microscopy to gain an appreciation of the movements to be explained. Electron microscopic examination of fixed critical-point dried cells was carried out to determine the three dimensional distribution of the filamentous structure associated with the cells' moving components. Thin filaments (4-7 nm in diameter) were associated with movements of the cell surface and intermediate-sized filaments (7-11 nm) were associated with the movements of organelles. Both sets of filaments had attached to them tiny 'node-like' structures with fine tails. These structures are frequently joined tail to tail to form 'dumb-bell' shaped structures.

Determination of the distribution of F-actin containing filaments was carried out with heavy meromyosin (HMM) and DNAase I. After giving the glycerinated/myosin-extracted cells a gentle pre-treatment with trypsin, it was shown electron microscopically that virtually all filaments, both thin and intermediate labelled clearly with HMM, this indicating a content of F-actin. This conclusion was supported by the results of treating cells with DNAase I (known to specifically depolymerize F-actin)

since this treatment caused a progressive depolymerization of both thin and intermediate filaments.

Determination of the form and localization of cytoplasmic myosin was carried out by treating cells with glycerol/myosin-extracting solutions and comparing the resultant cell fine structure with that of the normal (unextracted) cell. Electron microscopy of normal cells failed to show any myosin thick filaments. When cultured cells were treated with solutions known to extract myosin (here confirmed by SDS polyacrylamide gel studies) the effect was to remove the nodes with fine tails and 'dumb-bell' shaped structures normally present throughout the cytoplasm. These results suggested that the nodes with fine tails and 'dumb-bell' shaped structures represent cytoplasmic myosin in some highly disperse form. Support for this idea came from model studies with skeletal muscle actin and myosin, mixed together in a molar ratio equivalent to that found in non-muscle cells. When examined electron microscopically, the resultant actin/myosin filamentous complex bore close resemblance to the filamentous arrays found in cells.

Preliminary electron microscopic and biochemical investigations of the structure and molecular makeup of the intermediate filaments of chicken gizzard smooth muscle were undertaken to see whether these filaments also contain F-actin. The results showed that although these contained, in addition to a 55,000 dalton protein, significant quantities of actin, this actin was present in some non-filamentous form.



Overall, the various findings of the present work are consistent with the view that the movements of chick embryo connective tissue cells are brought about by actomyosin interactions involving filamentous actin, which is widespread throughout all levels of the cytoplasm, and myosin in the form of monomers and dimers.

## CHAPTER 1 - GENERAL INTRODUCTION



"The ability to move with other organisms  
to internal or external stimuli is without  
any doubt one of the most striking characteristics  
of living organisms" (Kornik, Stachem and  
Wohlfaeth-Buchmann, 1973).

The ability to move is a feature not only of  
multicellular organisms but also of single cell  
organisms. Indeed the ability of a multicellular  
organism to move is entirely dependent on the movement  
of its constituent cells (skeletal muscle cells).

## CHAPTER 1 - GENERAL INTRODUCTION

to illustrate that all of the organism's constituent  
cells, including its non-muscle cells, have the capacity  
to move. Moreover, cell motility is not an exclusive  
property of the animal kingdom, but it includes the plant  
kingdom as well. This illustrates that motility is an  
important fundamental property of all living cells.

Unfortunately, apart from the contraction of skeletal  
muscle cells, little is known about the mechanisms  
involved in the movement of various other types of cells.  
The major aim of this thesis is to provide a  
contribution towards the understanding of the molecular  
basis of the movements of non-muscle cells.

*"The ability to react with active motions to internal or external stimuli is without any doubt one of the most striking characteristics of living organisms"* (Komnick, Stockem and Wohlfarth-Bottermann, 1973).

The ability to move is a feature not only of multicellular organisms but also of single cell organisms. Indeed the ability of a multicellular organism to move is entirely dependent on the movement of its constituent cells (skeletal muscle cells). Various examples are cited below (see under Cell migration) to illustrate that all of the organism's constituent cells, including its non-muscle cells, have the capacity to move. Moreover, cell motility is not an exclusive property of the animal kingdom, but it includes the plant kingdom as well. This illustrates that motility is an important fundamental property of all living cells. Unfortunately, apart from the contraction of skeletal muscle cells, little is known about the mechanism involved in the movement of various other types of cells. The major aim of this thesis is to provide a contribution towards the understanding of the molecular basis of the movements of non-muscle cells.

### Type of Cell Movements

The movements of cells can be broadly classified into (a) Cell migration and (b) Subcellular motility. The latter can be further divided into surface movements of the cell membrane and movements within the cytoplasm. A highly specialized form of cell movement is skeletal muscle contraction.

(a) *Cell migration*: Cell migration takes a number of forms and many examples can be cited to illustrate this phenomenon. A bacterium propels itself by rapidly rotating its flagella. Other unicellular organisms, such as Paramecium, move by beating their cilia. Amoebae migrate by means of extending and retracting pseudopodia. This type of movement is also found in the cells of primitive multicellular metazoans where amoeboid migration of mesodermal cells towards food particles captured in the pseudocoelom is one aspect of their feeding behaviour.

In higher organisms, cell migration is a commonly observed phenomenon. Migration of cells takes place in physiological as well as pathological conditions. Cellular migrations play a crucially important role in morphogenesis and development of embryos (Spooner *et al.*, 1973). Examples of cell migration in pathological conditions are chemotactic movements of leukocytes (Florey, 1964), epithelial cell migration in wound healing (Florey and Jennings, 1964; Gibbins, 1976), platelet contraction following thrombus formation (Behnke, Kristensen and Nielsen, 1971) and invasion of normal tissues by tumour cells (Berenblum, 1964). Accordingly, the locomotion of cells in pathological events

is of great importance.

The migration of normal and tumour cells has also been observed in vitro by numerous authors since the early cell culture studies of Lewis and Lewis (1924). The advantage of this approach is that the cellular and subcellular movements can be examined in fine detail.

(b) *Subcellular motility*: Broadly, this can be subdivided into two separate categories:

1. Surface movements involving the cell membrane:

Examples of these movements are pseudopodium formation, phagocytosis, pinocytosis, exocytosis and membrane ruffling including the formation and retraction of fan-shaped extensions of the cell membrane and microvilli. These movements commonly involve only localized regions of the cell surface. As in the case of whole cell migration, surface movements are significant in physiological as well as pathological situations.

2. Movements deep within the cytoplasm: The most striking example of cytoplasmic movement is found in primitive green algae such as Nitella and other Characeae. Together with the endoplasm, particulate organelles of the cytoplasm are transported, along seemingly straight pathways in a constant streaming motion, at velocities of approximately 50 $\mu$ m/sec (Williamson, 1975). Another obvious example for this type of movement is found in the slime mould, Physarum polycephalum, in which reversible shuttle streaming in the veins is both spectacular and rapid. It serves as a means of transporting nutrients and is thought to play an important role in moving the



whole organism (Komnick, Stockem and Wohlfarth-Bottermann, 1973). Streaming movements have also been observed in some free-living animal cells such as Paramecium.

Cytoplasmic movements less rapid and more discriminatory, since they appear to involve movement of individual organelles rather than bulk cytoplasmic streaming, have for long been observed in cultured vertebrate cells (Lewis and Lewis, 1924; Frederic and Chevremont, 1952; Buckley, 1964; Buckley and Porter, 1967; Rebhun, 1972). The non-random nature of these organelle movements has been fully documented by Freed and Lebowitz (1970) and Rebhun (1972). Although movie records of these organelle movements in cultured cells illustrate this point, the non-random nature of the movements is most dramatically illustrated in the movement of organelles within the axonic processes of neurones (Pomerat *et al.*, 1967; McMahan and Kuffler, 1971; Kirkpatrick, 1971).

(c) *Contraction of muscle cells:* As an evolutionary end result, muscle cells exhibit a type of movement which is unique. As is well documented (Huxley, 1969), the muscle cell undergoes intermittent contractile (i.e. active shortening) movements which involve the whole cell. In these movements, force generation takes place in only one direction. By contrast, in non-muscle cells, movements mostly involve localized regions of the cell and these movements occur in three directions.

## Mechanisms of Cell Motility

Four mechanisms of cell motility have been proposed and investigated to date. These are:

- (1) The bacterial flagellar system which involves proteins such as flagellin (53,000 daltons) and a number of, as yet, undefined proteins involved in a proton-driven motor which rotates the flagella (Hilmen and Simon, 1976).
- (2) The myoneme system of stalked ciliates where the stalks are contractile by virtue of a rubber-like organelle which contracts in response to  $\text{Ca}^{2+}$  (Amos, 1978).
- (3) The tubulin-dynein system, which involves an interaction between microtubules and dynein, an ATPase. This system has been demonstrated to be the underlying mechanism for the beating of cilia and the movement of sperm-tails (Summers and Gibbons, 1971; Olmsted and Borisy, 1973).
- (4) The actin-myosin contractile system of muscle cells (Huxley, 1969) which will be discussed below.

The main concern of the present work is the subcellular movements exhibited by vertebrate non-muscle cells, which lack both flagella and cilia. Hence, the first two of the four mechanisms referred to above are not relevant to this study and will not be discussed further.

Microtubules probably play a vital role in chromosome movement during cell division and in the organelle motions which take place in the perikaryons and axons of neurones (Porter, 1976). This conclusion was arrived at because microtubules, along with neurofilaments,



form the major structural components of axons (Wuerker, 1970). Moreover, in the ventral nerve of the crayfish, neurofilaments are absent and only microtubules are present. Since cytoplasmic streaming is nevertheless observed in these axons, Porter (1976) argues that microtubules play the key role in cytoplasmic movements of neurones. In other than neurones, however, colchicine-treated cells (which have lost their microtubules) continue to exhibit active movements (Goldman and Knipe, 1973). Moreover, as it will be shown later (Chapter 3), the veil-like extensions of cultured chick embryo connective tissue cells, which show very rapid movements, are free of microtubules. Accordingly, although it seems that in certain kinds of movements, such as streaming within axons and movements of chromosomes during cell division, microtubules play a vital role, their general role in other aspects of vertebrate cell motility remains uncertain (Komnick, Stockem and Wohlfarth-Bottermann, 1973).

The next possible mechanism which may explain cell motility is an actin-myosin 'contractile' system similar to that of skeletal muscle. Loewy (1952) first observed that an extract obtained from Physarum and actomyosin extracted from muscle had many properties in common (e.g. contraction upon addition of ATP). Subsequently, as had earlier been shown with glycerinated muscle cells (Hanson and Huxley, 1955), glycerinated non-muscle cells were shown to contract in the presence of  $Mg^{2+}$  and ATP (reviewed by Arronet, 1973). Later, actomyosin isolated from platelets (termed 'thrombosthenin') was found to

contract under similar conditons; subsequently thrombosthenin was separated into its constituent actin and myosin fractions (Bettex-Galland, Portzehl and Lüscher, 1962). The purification of actin from Physarum (Hatano and Oosawa, 1966) was a turning point in demonstrating that actin-myosin based contractile systems can occur in non-muscle cells; electron micrographs of the negatively stained filaments obtained showed, without any doubt, the familiar double-helical structure of F-actin. According to Huxley (1976) this was the first really convincing evidence for an acto-myosin based contractile system in non-muscle cells. A second very important discovery was that of Ishikawa, Bischoff and Holtzer (1969) who showed, in a variety of glycerinated cell types, that heavy meromyosin can label certain (thin) filaments to give a characteristic arrowhead pattern similar to that originally observed in negatively stained preparations of HMM-decorated rabbit muscle F-actin (Huxley, 1963).

These striking discoveries encouraged workers to believe that most of the movements in non-muscle cells are brought about by an actin-myosin based contractile system analogous to that of skeletal muscle. It was thought that this actin-myosin interaction must have developed originally in the course of evolution in relatively simple cellular systems and that the mechanism used by muscle cells represents a highly specialized elaboration of the basic mechanism (Huxley, 1976). At this juncture it is relevant to describe briefly the sliding-filament mechanism,

over the myosin filaments. The mechanical force for sliding is generated by a series of changes:

involving actin and myosin, which has been shown to be responsible for active force generation (i.e. contraction) in skeletal muscle (Huxley and Hanson, 1954; Huxley, 1969).

#### The Molecular Basis of Skeletal Muscle Contraction

The basic functional unit of the contractile apparatus in skeletal muscle is called a sarcomere. This is the structure which, repeated many times in series and in parallel in each muscle cell, accounts for the striations seen microscopically. Each sarcomere consists of interdigitating sets of thin and thick filaments (Fig. 1). Each thin filament is made up primarily of two strands of globular actin molecules helically wound to form an F-actin filament. In addition to F-actin, these filaments contain the regulatory proteins tropomyosin and troponin. The thick filaments are composed of bi-polar assemblies of myosin molecules together with some minor accessory proteins. The two sets of actin and myosin filaments have opposite polarity to each other in the sense that when they interact with each other to produce contraction, the actin filaments on each side of the sarcomere are drawn unidirectionally along the myosin filaments towards the centre of the sarcomere. In cross-section, it is evident that the actin filaments are arranged in a hexagonal array surrounding the myosin filaments (Fig. 1).

Contraction of muscle takes place due to synchronous shortening of sarcomeres. As indicated above, this shortening is caused by sliding of the actin filaments over the myosin filaments. The mechanical force for sliding is generated by a series of changes:

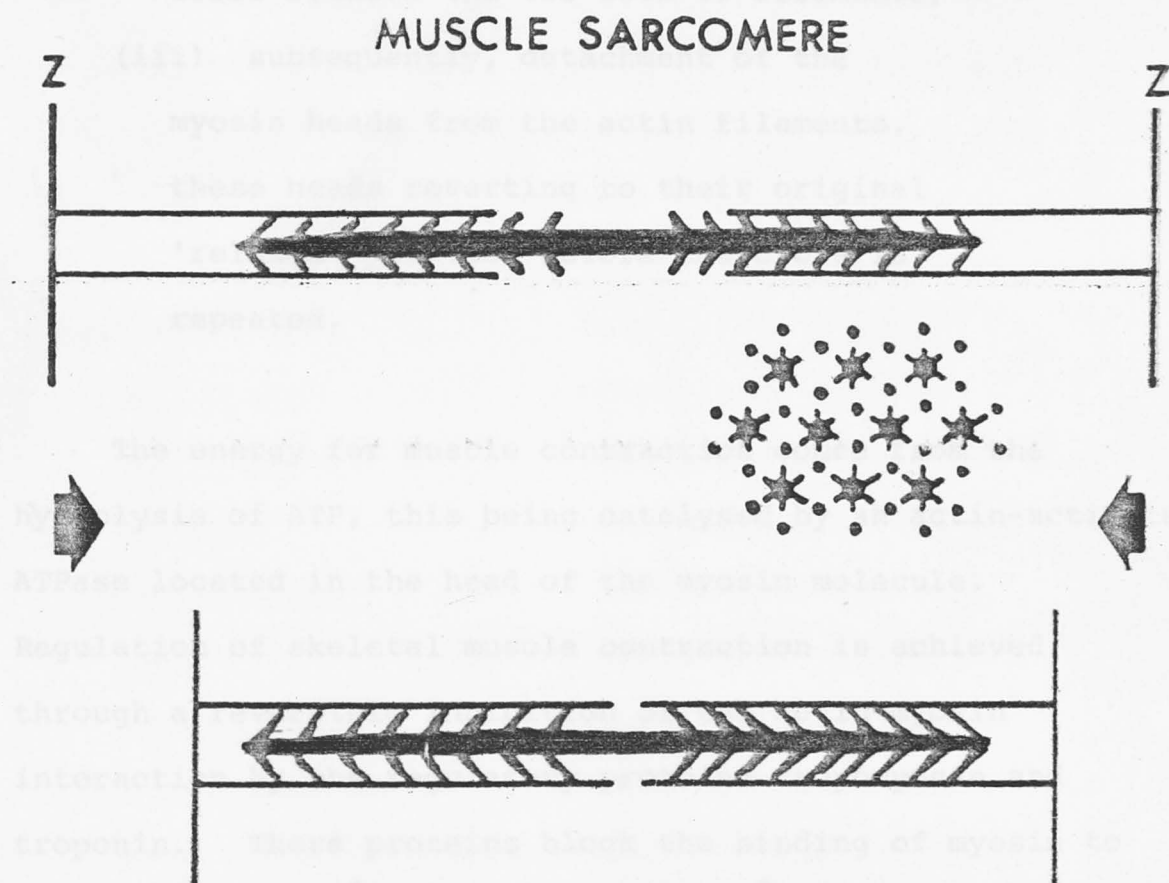


FIGURE 1. Line drawings of a striated muscle sarcomere. From the top, longitudinal section of an extended sarcomere, cross section through a region of thick and thin filament overlap, longitudinal section of a contracted sarcomere.



- (i) attachment of the heads of the myosin molecules to the actin filaments;
- (ii) a conformational change in the myosin heads which exerts an active shearing force between the two sets of filaments;
- (iii) subsequently, detachment of the myosin heads from the actin filaments, these heads reverting to their original 'relaxed' position before the cycle is repeated.

The energy for muscle contraction comes from the hydrolysis of ATP, this being catalysed by an actin-activated ATPase located in the head of the myosin molecule. Regulation of skeletal muscle contraction is achieved through a reversible inhibition of the actin-myosin interaction by the regulatory proteins tropomyosin and troponin. These proteins block the binding of myosin to actin when the  $\text{Ca}^{2+}$  level is below  $10^{-7}\text{M}$ . When its concentration rises above  $10^{-5}\text{M}$ ,  $\text{Ca}^{2+}$  binds to troponin and the inhibitory effect of the troponin-tropomyosin complex on the actin-myosin interaction is removed (reviewed by Gergely, 1976).

A possible analogy between muscle contraction and non-muscle cell motility (Huxley, 1963) encouraged investigators to look for actin and myosin in various types of non-muscle cells.

#### Properties of Non-muscle Actin

There is a wealth of biochemical and ultrastructural

information available about actin isolated from non-muscle cells (reviewed by Pollard and Weihing, 1974; Clarke and Spudich, 1977; Korn, 1978). Actin has been found in various non-muscle cells and it is considered to be one of the major cellular proteins, comprising some 5-10% of the total protein content of most cells (Pollard and Weihing, 1974). The following are a few examples of actin-containing non-muscle cells illustrating what appears to be a ubiquitous presence of actin in eukaryotes:

<u>Cell Source</u>	<u>Reference</u>
<u>Acanthamoeba</u>	Gordon, Eisenberg and Korn (1976)
<u>Physarum</u>	Owaribe and Hatano (1975)
<u>Dictyostelium</u>	Cooke <i>et al.</i> (1976)
<u>Chara</u>	Williamson (1974)
<u>Limulus</u> sperm	Tilney (1975)
Human erythrocytes	Sheetz, Painter and Singer (1976)
Human blood platelets	Gallagher, Detwiler and Stracher (1976)
Rabbit macrophages	Hartwig and Stossel (1975)
Rat brain tissue	Le Beux and Willemot (1975)
Cultured chick embryo fibroblasts	Yang and Perdue (1972) Bray and Thomas (1975)
Tumour cell line	Gabbiani, Trenchev and Holborow (1975)

The non-muscle actins purified from various tissues are similar to their muscle counterparts in most physical and chemical properties (reviewed by Pollard and Weihing, 1974). These properties include:



- (i) Apparently identical molecular weight, ( $\sim 42,000$  daltons);
- (ii) Ability to form characteristic double helical filaments of 5-7 nm in diameter;
- (iii) Ability to bind HMM to give arrowhead complexes;
- (iv) Formation of paracrystalline arrays when the  $Mg^{2+}$  level is increased above  $\sim 15$  mM;
- (v) Ability to specifically activate myosin ATPase. Here, however, there appears to be some difference because the level of activation by non-muscle actins is 3-5 times less than that of muscle actin;
- (vi) All actins contain the amino acid  $N^{\gamma}$ -methylhistidine (Clarke and Spudich, 1977).

Although there are a large number of similarities, there are several differences between actins of muscle and non-muscle origin. These include:

- (i) Peptide mapping has shown that actins from different sources (e.g. brain, heart, platelets muscles) differ from each other by at least one amino acid. The work of Storti and Rich (1976) appears to show that different genes are involved in the synthesis of actin in different tissues.
- (ii) Two dimensional gel electrophoresis has shown that actin can be distinguished into  $\alpha$ ,  $\beta$ , and  $\gamma$  types with slightly

different isoelectric points (Lazarides and Balzer, 1977). Skeletal muscle actin is of the  $\alpha$  type.  $\gamma$ -actin is present in chicken gizzard smooth muscle. Most of the non-muscle cell types contain various proportions of both  $\beta$  and  $\gamma$  actins.

- (iii) Bray and Thomas (1976) found that when actin from chick embryo fibroblasts is released by gentle procedures, nearly 50% of it failed to polymerize under conditions known to completely polymerize muscle actin.

Despite these minor differences, the structural and functional similarities which exist amongst various actins are remarkable and it appears that the actin molecule is one of the most highly conserved molecules in nature (Clarke and Spudich, 1977).

#### Properties of Non-muscle Myosin

Although the list is not as extensive as for actin, myosin has been found in a great variety of non-muscle cells. The quantity of myosin in non-muscle cells is very low (0.3-1.5% of total cell protein) (Pollard and Weihing, 1974). Thus the molar ratio of myosin to actin in non-muscle cells is  $\sim 1:100$  (cf. skeletal muscle where the ratio is 8:1). The following are a few examples of cells from which myosin has been purified:

<u>Cell Source</u>	<u>Reference</u>
<u>Physarum</u>	Kessler, Nachmias and Loewy (1976)
<u>Acanthamoeba</u>	Pollard <i>et al.</i> (1976)
Cultured cells (fibroblasts)	Ostlund, Pastan and Adelstein (1974)
Rabbit macrophages	Stossel and Hartwig (1976)
Human platelets	Niederman and Pollard (1975b)
Secretory tissue (hog salivary and pituitary glands)	Ostlund, Leung and Kipnis (1978)
Rat brain	Burridge and Bray (1975)

Non-muscle myosins share with muscle myosins many essential functional and structural properties (Pollard and Weihing, 1974; Clarke and Spudich, 1977). These are as follows:

- (i) All myosins have an inherent ATPase activity which is stimulated by actin;
- (ii) They all bind physically to actin filaments, as demonstrated by electron microscopy and viscosity measurements;
- (iii) With the exception of Acanthamoeba myosin, all myosin molecules are composed of two heavy polypeptide chains, each having a molecular weight of about 200,000 daltons (200K) and two light chains of about 21K and 17K;
- (iv) Under physiological ionic conditions, purified myosin from non-muscle cells will form bipolar thick filaments in vitro;
- (v) The sizes and shapes of individual molecules

of non-muscle myosin closely resemble those of skeletal muscle myosin (Elliott, Offer and Burridge, 1976).

These similarities are accompanied by some differences between myosins of muscle and non-muscle cell origin.

These differences are:

(i) Even though the non-muscle myosin-ATPase activity is stimulated by actin, the increase in activity is only  $\sim 10$ -fold compared with skeletal muscle myosin, where the increase is  $\sim 50$ -fold;

(ii) The molecular weights of myosins from different non-muscle cells are similar but not identical. For example, in the case of Acanthamoeba, the molecular weight of myosin is only 180,000 daltons (cf. skeletal muscle myosin's molecular weight of 500,000 daltons);

(iii) Burridge (1976), studying the cleavage pattern of various myosins by cyanylation, found that myosins can be grouped into six types (on the assumption that the cleavage pattern reflects true differences in the primary sequence). He attributed the difference between brain and platelet myosins to the different motility behaviour appropriate to neurones and platelets.



The functional significance of these differences between myosins is not yet clearly known. However, it is known that there are significant differences even between myosins derived from various types of muscle (e.g. 'fast' and 'slow' contracting muscles (Bárány and Close, 1971)). Accordingly, it is not surprising that there are notable differences between the muscle and non-muscle myosins and also between the non-muscle myosins themselves, depending on the type of cell. Nevertheless, the evidence that myosin is involved in the active force-generating mechanism for the movements of non-muscle seems altogether convincing (Pollard, 1976b).

#### Localization and Form Determination of Contractile Proteins in Non-muscle Cells

Apart from the characterization of isolated actin and myosin, the other important step which must be taken to support the concept of an acto-myosin based contractile system in non-muscle cells is the localization and the determination of the form taken by actin and myosin within these cells. Compared with the wealth of biochemical and ultrastructural information available about isolated actin and myosin from non-muscle cells, relatively little progress has been made in this field. This may be due in part to the lack of a relatively stable and highly organized structure such as the sarcomere found in striated muscle. Anyone expecting to find in non-muscle cells a structural organization of actin and myosin directly comparable to that of skeletal muscle should bear in mind the pronounced differences in the character of the movements exhibited



by muscle and non-muscle cell types (Buckley and Raju, 1976). One would then expect these motility function differences to be based on a markedly different structural arrangement of the prime motility proteins.

(a) *Localization of actin filaments:* Attempts have been made to localize cellular actin in situ using four different approaches:

(i) Morphological comparisons, that is, attempting to identify F-actin filaments in non-muscle cells purely on the basis of morphological characteristics, especially that of filament diameter in relation to that of the thin actin-containing filaments of skeletal muscle.

(ii) Treating glycerinated cells with heavy meromyosin (HMM) to see which filaments label with HMM (Ishikawa, Bischoff and Holtzer, 1969).

(iii) Immunofluorescence localization of actin filaments, using fluorescein-labelled actin-specific antibodies (Lazarides and Weber, 1974; Lazarides, 1976) or fluorescein-labelled HMM (Goldman, 1975; Herman and Pollard, 1978).

(iv) Ultrastructural observation of cells treated with low ionic strength (actin depolymerizing) solutions (Pollard and Weihing, 1974).

Of the four techniques, HMM labelling and immunofluorescence antibody staining have been widely applied. HMM labelling studies have been conducted in different types of tissue cells such as macrophages, platelets, fibroblasts, epithelial cells, neurones, eggs and spermatocytes (for review, see Pollard and Weihing, 1974). To date this is the most specific histochemical technique for actin identification. The immunofluorescence studies have been extremely valuable in providing an overview of the intracellular distribution of actin. However, their precision in localizing proteins is limited to the resolution limits of the light microscope.

(b) *Localization of myosin:* Localization of cellular myosin in situ has always met with problems because of the low concentrations of myosin within non-muscle cells (Pollard *et al.*, 1976). Attempts to identify myosin within the cells have been carried out using the following approaches:

- (i) Direct observation of fixed sectioned cells, aiming to find bi-polar myosin thick filaments (Niederman and Pollard, 1975).
- (ii) Immunocytochemical localization using myosin-specific antibodies (Weber and Groeschel-Stewart, 1974; Fujiwara and Pollard, 1976).

Bi-polar thick filaments have been reported in only a very few cell types and in a number of instances, these cells were specially pre-treated before fixation (reviewed

by Pollard *et al.*, 1976). These authors also remarked that bi-polar myosin filaments in non-muscle cells are few in number and small in size and are therefore very difficult to identify positively within the cytoplasm.

With respect to the immunofluorescence approach, even though the techniques can be highly specific, the accuracy of localization is severely limited by the resolving power of the light microscope.

Thus, overall, the knowledge we have on the ultrastructural localization of actin and (to a greater extent) myosin is limited. In the case of F-actin (the filamentous form of which is necessary for an actin-myosin shear force-generating system), its distribution appears limited to the submembranous cortical bundles in well spread cells and to the submembranous filamentous network in rounded cells (Goldman *et al.*, 1976). Such a distribution could not explain the movements that take place in the deeper regions of the cytoplasm involving changes in cell shape and organelle movements. As mentioned earlier, in the case of myosin localization the situation is worse since it is still unclear what form myosin takes in non-muscle cells.

Accordingly, there is a need for a thorough ultrastructural investigation to determine the form taken by F-actin and myosin within non-muscle cells and, broadly speaking, this thesis is intended to fulfil this need.

#### Specific Aims of this Thesis

- (1) To study the three dimensional architecture of

cultured non-muscle cells in order to understand the form and distribution of the filamentous elements which are topographically related to the moving parts of the cells.

(2) To study the distribution of F-actin within non-muscle cells.

(3) To study the form and distribution of myosin in non-muscle cells.

#### Technical Approaches

To achieve these aims the following approaches were taken:

(1) The three dimensional arrangement and distribution of filaments within non-muscle cells were studied by using improved methods of fixation and critical-point drying in conjunction with stereoscopic viewing of stereo pairs of electron micrographs (Buckley and Raju, 1976).

(2) The distribution of F-actin was examined by:

(i) HMM labelling of glycerinated cells

using conventional methods (Ishikawa, Bischoff, and Holtzer, 1969) as well as

a modified HMM labelling procedure developed

by Buckley, Raju and Stewart (1978).

(ii) The selective depolymerization and

extraction of actin-containing filaments

from cells using DNAase I, a highly

specific actin-depolymerizing agent.

(iii) Comparing the fine structure of the



filamentous system of cells with purified actin from skeletal muscle, polymerized and subjected to the same treatments (mentioned in (i) and (ii) above) applied to cells.

(3) The form and distribution of myosin was studied by:

(i) Glycerinating and extracting the cells with myosin-extracting solutions to determine which structures normally associated with actin filaments were removed from the myosin-extracted cells.

(ii) Studying the structure of purified molecules of muscle myosin and myosin synthetic thick filaments processed for electron microscopy by the same procedure used to study the cells.

(4) A preliminary analysis of chicken gizzard smooth muscle intermediate filaments was also undertaken to see whether there is any similarity in the intermediate filaments of chick embryo connective tissue cells and chicken gizzard smooth muscle cells.

#### Experimental Model

Cultured chick embryo connective tissue cells of the fibroblastic type were selected as an experimental model because:

- (1) They are non-muscle in type.
- (2) They are highly active in culture, exhibiting prolific



cellular and subcellular movements.

(3) These cells spread out well in culture and hence it is easy to study the ultrastructure in most areas of the cell, even in whole mount preparations.

(4) The cells are easy to culture. One needs only small quantities of material and short incubation times.

The various experiments conducted in this thesis involved studies of chick embryo connective tissue cells in culture, rabbit skeletal muscle and chicken gizzard smooth muscle. Microscopical and gel electrophoretic techniques were common to all three. Hence this chapter is divided into 4 main categories covering each of the above and further subdivided as shown in the following:

Techniques related to Cultured Chick Embryo Connective Tissue Cells

- (a) Culturing of cells;
- (b) Fixation of normal cells;
- (c) Glycerination;
- (d) Myosin extraction;
- (e) Heavy meromyosin (HMM) labelling of cells;
- (f) DMSO treatment of cells.

Techniques related to Actin and Myosin Isolated from Rabbit Skeletal Muscle

- (a) Preparation of acetone-powder of actin;
- (b) Purification of actin from acetone-powder;
- (c) Isolation and purification of myosin;
- (d) Preparation of heavy meromyosin;
- (e) Model experiments with actin and myosin.

Techniques related to Chicken Gizzard Smooth Muscle

- (a) Isolation of intermediate filaments;
- (b) DMSO treatment of intermediate filaments;

The various experiments conducted for this thesis involved studies on chick embryo connective tissue cells in culture, rabbit skeletal muscle and chicken gizzard smooth muscle. Microscopical and gel electrophoretic techniques were common to all three. Hence this chapter is divided into 4 main categories covering each of the above and further subdivided as shown in the following:

Techniques related to Cultured Chick Embryo Connective Tissue Cells

- (a) Culturing of cells;
- (b) Examination of normal cells;
- (c) Glycerination;
- (d) Myosin extraction;
- (e) Heavy meromyosin (HMM) labelling of cells;
- (f) DNAase I treatment of cells.

Techniques related to Actin and Myosin Isolated from Rabbit Skeletal Muscle

- (a) Preparation of acetone-powder of actin;
- (b) Purification of actin from acetone-powder;
- (c) Isolation and purification of myosin;
- (d) Preparation of heavy meromyosin;
- (e) Model experiments with actin and myosin.

Techniques related to Chicken Gizzard Smooth Muscle

- (a) Isolation of intermediate filaments;
- (b) DNAase I treatment of intermediate filaments;

- (c) Trypsin treatment and HMM labelling of intermediate filaments.

#### Microscopical and Gel Electrophoretic Techniques

- (a) Light microscopy;  
 (b) Electron microscopy;  
 (c) Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis.

## Techniques related to Cultured Chick Embryo Connective Tissue Cells

(a) *Culturing of cells:* The culturing of chick embryo connective tissue cells was carried out in chambers similar to those described by Rose *et al.* (1958). These chambers are well suited for the light microscopic study of the primary cultures of embryonic cells used in the present studies.

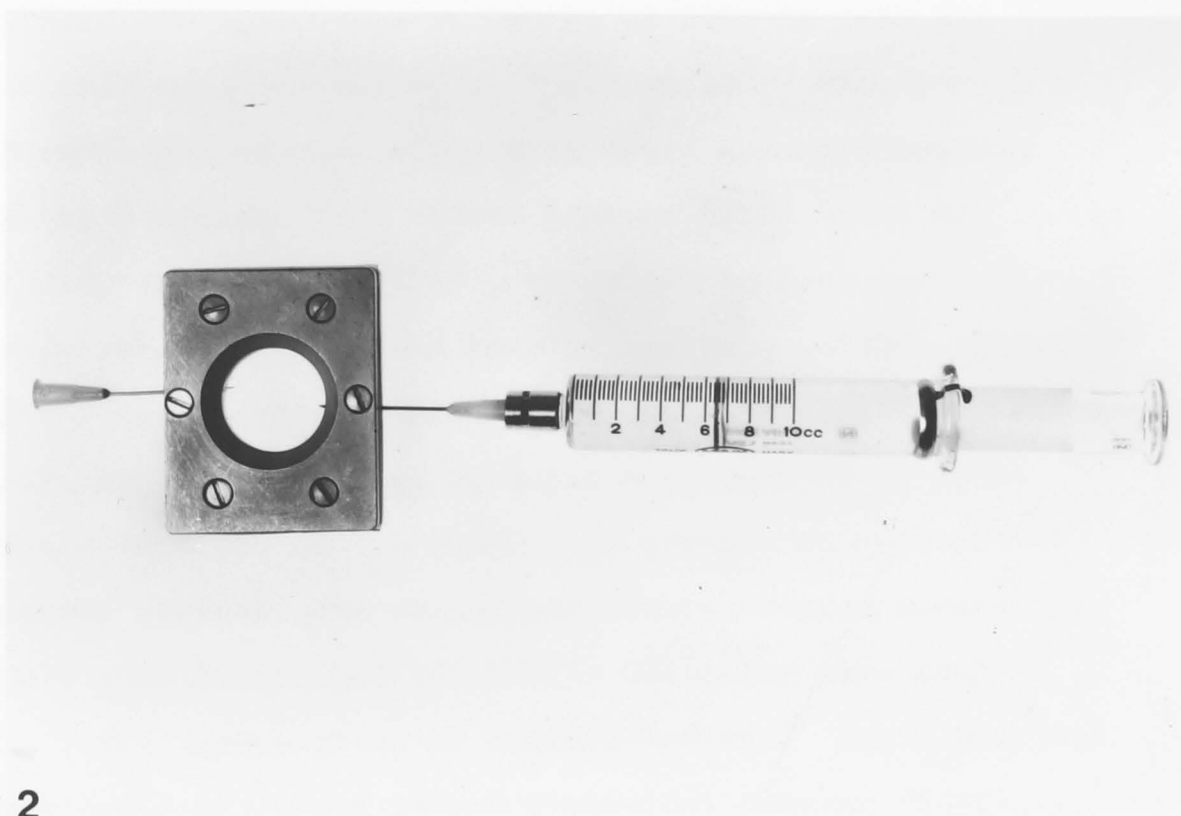
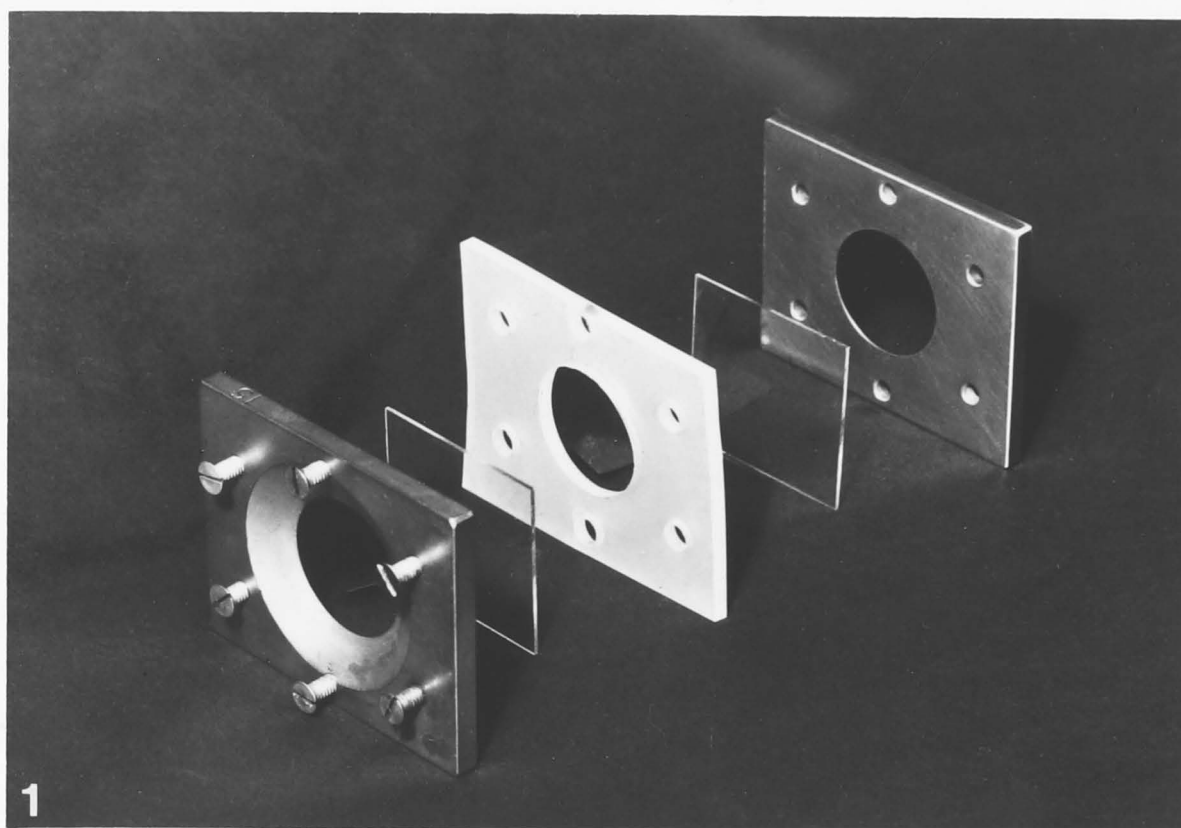
1. *Rose Chamber:* As shown in Fig. 1, the Rose chamber is a sandwich-type assembly consisting of two metal plates enclosing a glass coverslip on which cells grow, a silicone rubber gasket which, acting as a spacer, provides a well for culture medium and a very thin polystyrene coverslip through which the respiratory gases, oxygen and carbon dioxide, can diffuse. The metal plates were machined from brass and electroplated with nickel. The gaskets were cut from 1/8" thick sheets of non-toxic translucent silicone rubber (RWAY Synthetic Producers Div., Reise Manufacturing Corp. Little Falls, N.J., U.S.A.). The glass coverslips were 38 x 38 mm, No 2 thickness (Chance Bros. Ltd., Birmingham, England) and were either formvar-coated or formvar-and carbon-coated as described below. The gas-diffusible coverslips were cut from a 0.01" thick polystyrene sheet ('Polyflex', Sidaplex, Gentbrugge, Belgium). The components of this assembly (metal plates, coated glass coverslips, silicone rubber and polystyrene coverslips) were assembled in that order and clamped together with six screws (Fig. 1).

2. *Formvar and Carbon Coating of Glass Coverslips:* The glass coverslips were coated with 0.7% (w/v) formvar



Fig. 1: A dismantled Rose chamber showing the two metal plates, glass coverslip, on which cells grow, a silicone rubber gasket, which, acting as a spacer, provides a well for culture medium and a very thin polystyrene coverslip through which respiratory gases, oxygen and carbon dioxide, can diffuse.

Fig. 2: The culture medium and isolated cells were injected into the chamber, using needles of 20 gauge size passed through the silicone rubber.



in chloroform. This coating was carried out by individually dipping previously washed and heat-sterilized coverglasses in the formvar solution for 5-10 secs before withdrawing them and allowing excess solution to drain off. During this process care had to be taken to prevent trapping of moisture by the formvar as it cooled during drying, as this resulted in cloudy films. This was achieved by holding the circular end of a heated brass block close to the glass coverslip as soon as it was taken out from the formvar solution. To prevent unwanted separation of the formvar film from the coverslip over the area covered by the silicone rubber gasket, a heated brass ring was placed over the outer margins of the coated coverslip for 1-2 secs. This did not affect the subsequent stripping of formvar from the central area covered by cultured cells. When required, formvar-coated coverslips were given a carbon coating using a Dynavac High Vacuum Coating Unit, Model CE 12/14S (Dynavac Pty. Ltd., Victoria, Australia). By experience it was found that an adequate coating of carbon ( $\sim 4-5$  nm thick) was obtained if carbon evaporation was carried out at  $10^{-6}$  mm Hg using a current of 30-50 mA for a duration of 1.5 mins. To produce an hydrophilic carbon surface, the carbon-and formvar-coated coverslips were glow discharged for 5 mins using the same machine.

3. Preparation of Culture Medium: Ham's nutrient mixture F-12 (Grand Island Biological Company (GIBCO)., Grand Island, New York, USA) instant culture medium powder was used to prepare the culture medium. The contents of one packet were dissolved in one litre of

glass-distilled water at room temperature using a magnetic stirrer. To this was added 0.5 ml of a 5% stock antibiotic solution (25 mg each of benzyl penicillin, streptomycin sulphate and neomycin sulphate dissolved in 500 ml deionized water). The pH of the medium was adjusted to 7.0 with 1.4%  $\text{NaHCO}_3$ . When the medium was completely dissolved, stirring was stopped and the medium was filtered through a 0.45 $\mu$  millipore filter and stored at 4°C in 90 ml lots in milk dilution bottles. When required for use, 10 ml of foetal calf serum (Commonwealth Serum Laboratories, Victoria, Australia) was added to 90 ml of medium. This culture medium (complete with serum) was warmed to 37°C in a water bath before being used for culture.

4. Culturing Techniques: Cultures were made from 10-12 day old chick embryos. Working with a laminar flow hood (Clemco Ultraviolet Products, Artarmon, N.S.W., Australia) and using aseptic techniques, the egg air-space was entered and the whole embryo was transferred to a Petri dish. The embryo was immediately decapitated and the head discarded. This was followed by removal of limbs and skin. The decapitated embryo was immersed in 10 ml of Hank's Balanced Salt Solution (HBSS) at pH 7.0. Using a dissecting microscope, fine pointed pairs of forceps and scissors, the embryo was eviscerated and the remaining tissue was transferred to a beaker and minced finely with small scissors. The minced tissue was then transferred to a 50 ml flask containing 0.2% trypsin ((DIFCO Laboratories, Detroit, Michigan, U.S.A.)); prepared as a 10% stock in 0.8% NaCl, 0.04% KCl, 5%

glucose, 0.006%  $\text{NaHPO}_4$  and 0.006%  $\text{KH}_2\text{PO}_4$ ) in 10 ml of HBSS. Then the flask was gently agitated for 20 mins using a shaker kept in a 37°C warm room. This helped the trypsin to penetrate the tissue fragments and loosen the cells effectively. Following trypsin treatment, the contents of the flask were transferred to a sterile 15 ml graduated centrifuge tube. Once the fragments settled, the supernatant trypsin solution was withdrawn gently with a syringe without disturbing the fragments, and replaced by 10 ml of culture medium at 37°C. Using a 20 ml syringe and 14 gauge needle with a blunt tip, the fragments, suspended in this complete culture medium, were very gently aspirated back and forth through the needle. Doing this slowly, while keeping them continually suspended to avoid compression from crowding within the needle and crushing between the end of the plunger and barrel of the syringe, the tiny tissue fragments were progressively reduced to smaller and smaller clumps over a period of 2 mins. As soon as a good yield of cells and microscopic clumps was obtained (indicated by the cloudiness of the suspension) the aspiration was stopped. The large unbroken fragments were allowed to settle to the bottom of the tube. The supernatant medium, containing cells and tiny cell clumps, was then drawn into the same syringe and transferred to a graduated centrifuge tube and centrifugation was carried out at low speed (1,000 rev/min) in a MSE 'Minor' bench top centrifuge for 4 mins, just sufficient to bring down the microscopic clumps and most of the intact cells. The supernatant, which contained mostly cell debris and few cells, was



discarded and the pellet, composed of intact cells and cell clumps, was resuspended in 5 ml of fresh culture medium. The harvested cells were injected into the cavities of Rose chambers which had already been filled with warmed ( $\sim 37^{\circ}\text{C}$ ) culture medium. Needles of 20 gauge size were passed through the silicone rubber of the culture chamber for injecting the medium as well as the cells (Fig. 2). Once chambers were seeded with cells they were stored, coverglass down, in a thermal incubator adjusted to  $37^{\circ}\text{C}$ , for 24 hours until the cells had adhered to and spread thinly on the formvar or formvar- and carbon-coated glass coverslip. A gas phase suitable for the culture was provided by gassing the incubator continuously with 5%  $\text{CO}_2$  in air.

(b) *Examination of normal cells:* After 24 hours of culturing, the Rose chambers were taken out from the thermal incubator and observed under a phase contrast light microscope (for light microscopy, see p.47 ). To obtain a clear view of the cells, the formvar from the outer surface of the glass coverslip was removed. Chambers containing a suitable concentration (neither too sparse nor too dense) of well spread healthy cells were selected for further use. The ultrastructure of normal cells was studied by critical-point drying (see p.48 in this chapter).

(c) *Glycerination:* Glycerination of cells in the presence of the standard salt solution of Huxley (1963) or of a modification of this (see below) was carried out

for the following purposes:

- (i) To make the plasma membrane of cells permeable so that organelles and soluble proteins would diffuse out.
- (ii) To enable extracting solutions (e.g. myosin-extracting solution) to permeate the cells and, where applicable,
- (iii) to label the residual filaments with heavy meromyosin (HMM).

1. Glycerol-salt solutions used: The glycerol/standard salt solution mixture contains equal volumes of glycerol (analytical grade, BDH Chemicals Ltd., Poole, England) and either Huxley's (Huxley, 1963) standard salt solution (0.1M KCl, 1mM  $MgCl_2$  and 6.7mM potassium phosphate buffer, pH 7.0, at 4°C) or a modified standard salt solution containing more magnesium. This was: 0.1M KCl, 5mM  $MgCl_2$  and 6.7mM potassium phosphate buffer, pH 7.0, at 4°C. Later it was found that the use of either 10-20mM Imidazole-HCl or 5mM Tris-HCl buffers at the same pH (instead of the phosphate buffer) gave equivalent results.

2. Glycerination procedure: The cells were glycerinated after 18-24 hours of culturing, when light microscopic examination showed that they were well spread out and healthy. The culture medium was aspirated from the chamber and replaced immediately with pre-cooled (4°C) glycerol-salt solution. Extraction was carried out for a period of 24 hours at 4°C. In some experiments, following this initial extraction with 50% glycerol, the cells were extracted with 25%, 12.5% and 5% glycerol in standard salt solution for 12 hours, 5 hours and 5 hours

respectively (Ishikawa, Bischoff and Holtzer, 1969). The glycerinated cells were again examined by light microscopy and only those chambers which still had well spread cells were selected for further uses, such as myosin extraction, HMM-labelling and DNAase I extraction. The ultrastructure of glycerinated cells themselves was studied either by critical-point drying or negative staining (see p.48 and 53 in this chapter).

(d) *Myosin extraction:* As an aid to elucidating the localization and form determination of myosin in cultured chick embryo connective tissue cells, extraction of myosin from the cells was carried out. The extraction procedure is based on the uncoupling of myosin from actin using pyrophosphate, thereby enabling myosin molecules to diffuse out of the cell.

The myosin extraction was carried out as a 'one-step' or a 'two-step' procedure. In the 'one-step' myosin extraction, glycerination and myosin separation were carried out at the same time. In the 'two-step' myosin extraction procedure, the cells were glycerinated in the presence of standard salt solution as described earlier (see p.31) and then extracted with the pyrophosphate-rich myosin extracting solution.

1. Composition of myosin extracting solution:

50% or 15-20% Glycerol	-	50% in the 'one-step' myosin extraction procedure, or 15-20% in the 'two-step' myosin extraction procedure. (to render the cells permeable)
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30mM Pyrophosphate  
(to uncouple myosin  
from actin)

0.5mM DTT (Dithiothreitol)  
(to protect the thiol groups)

0.7mM  $MgCl_2$   
(to preserve the F-actin  
filaments)

10mM Potassium phosphate buffer,  
pH 7.0 at 4°C.

2. Experimental procedure: In the 'one-step' myosin extraction, the culture medium of a 24 hour cell culture was aspirated from the culture chamber and replaced with the myosin-extracting solution pre-cooled to 4°C. The chambers were stored at 4°C for varying lengths of time, from 2 hours to several days.

In the 'two-step' myosin extraction procedure, the culture medium from the chamber was replaced with glycerol-standard salt solution and the cells were glycerinated as described earlier (see p.30) for 24 hours at 4°C. Following glycerination, the cells were extracted for myosin by replacing the glycerol-standard salt solution with the myosin-extracting solution. The extraction was carried out for 24 hours at 4°C.

After extraction, the chambers were dismantled and the cells thoroughly washed by rinsing the coverglass through Petri dishes of the extracting solution (without glycerol and pyrophosphate). Fixation and critical-point drying or negative staining was carried out as described below (see p.48 and 53 in this chapter).

(e) Heavy meromyosin (HMM) labelling of cells:

1. Without trypsin pre-treatment: HMM was obtained from myosin using the procedure of Lowey *et al.* (1969) (for



details of preparation see p.41 in this chapter). Either glycerinated, or glycerinated/myosin-extracted cells were used for HMM-labelling. Before use the culture chamber was dismantled and the coverglass with attached cells was washed in small Petri dishes, using 3-4 changes of standard salt solution (SSS) (Huxley, 1963). For HMM labelling, the SSS was drained off the coverglass and several drops of the stock HMM solution were applied directly to the cells. The treated cells were then stored at 4°C within a covered Petri dish (to prevent drying) for 12-24 hours. At the end of this period, the cells were washed for 10-15 mins in several changes of ice-cold SSS to remove glycerol and excess HMM. After washing, the cells were fixed and negatively stained (see p.53 in this chapter).

2. With trypsin pre-treatment: Glycerinated, myosin-extracted cells were treated with trypsin (TPCK 253U/mg) (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) at a concentration of 5µg/ml made up in 50% glycerol/50% SSS. The original solution from the culture chamber was aspirated and replaced by the trypsin solution. The trypsin treatment was carried out for 1-3 hours at 20°C. Higher concentration of trypsin or significantly longer times (e.g. >5 hours) resulted in the destruction of all filaments. When the trypsin treatment was finished, the culture chamber was dismantled and the coverglass with attached cells was washed in small Petri dishes, using 3-4 changes of 50% glycerol/50% SSS at 4°C. To ensure adequate washing away of the trypsin, the cells were left in the final Petri dish for 1 hour. HMM labelling and



electron microscopic procedures were carried out as in the previous case (i.e. HMM labelling without trypsin pre-treatment).

(f) *DNAase I treatment of cells:* DNAase I treatment was carried out on either glycerinated, or glycerinated/myosin-extracted cells. The culture chamber was dismantled and the coverglass with attached cells was washed in SSS and, while still immersed, small round areas of formvar were stripped from the coverglass, floated cell side down on the surface of the solution and grids applied (for details of application of grids, see p.49 in this chapter).

Treatment with DNAase I was carried out by floating the grids, cell side down, on droplets containing 2 mg/ml of DNAase I (type DN-CL) (Sigma Chemical Company, St. Louis, Mo., U.S.A.) in SSS, placed on sheets of parafilm in a moist chamber. Cells were treated with DNAase I for 2 hours at room temperature followed by overnight at 4°C. Control cells were treated in an identical manner, except that they were floated on droplets of SSS which did not contain any DNAase I. The cells were washed in SSS for 30 mins prior to fixation and negative staining or critical-point drying (for details of electron microscopic procedures, see p. 48 and 53 in this chapter).

Techniques Related to Actin and Myosin Isolated from  
Rabbit Skeletal Muscle

(a) *Preparation of acetone-powder of actin:* The purification of actin can be divided into two stages. The first stage involves preparation of an acetone-powder which can be stored for up to several months. The second stage involves further purification of acetone-powder to obtain homogeneous actin.

1. Killing and skinning the rabbit: The rabbit was held firmly face-down on an operating table with front and hind limbs pulled apart by an assistant. Holding the ear lobes with the left hand, a couple of firm and swift blows were given to the back of the rabbit's neck to stun the animal. The stunned animal was killed by incising the carotid arteries. The body of the animal was pressed intermittently to pump out as much blood as possible.

A shallow incision was made circumferentially around the trunk and the skin progressively reflected. To facilitate removal of the skin from the hind limbs, incisions were made around both legs at the ankle.

2. Isolation of back and leg muscles: After skinning, the animal was laid on the operating table. Two long incisions were made close to the vertebral column on either side. The back muscles were cut from their anterior attachments and, by working the scalpel (with a sharp blade) close to the vertebral column and ribs, the whole muscles were isolated and freed from their posterior attachments. Immediately the muscles were wrapped in plastic 'Glad wrap' and immersed in a bucket

of crushed ice crystals. The muscles were stripped of connective tissue as far as possible during isolation. A median incision was made on the thigh on both sides and the leg muscles were pulled by hand and freed from their attachment sites. As far as possible all the white muscles were isolated omitting the red muscles. The leg muscles were also wrapped in plastic and immersed in the bucket of ice. The muscles were removed from the ice bucket after 30 mins and placed on a glass plate. The remaining connective tissue was completely removed from the muscle. Cooling the muscles helped the process of removing the connective tissue. The trimmed muscle pieces were minced once with a pair of scissors and wrapped in plastic and weighed.

3. Extraction with Guba-Straub myosin-extracting solution: The minced muscle pieces were extracted with 3 volumes of freshly prepared Guba-Straub's myosin-extracting solution. This consists of 0.3M KCl, 0.15M potassium phosphate buffer at pH 6.5 (Szent-Györgyi, 1947). The contents were stirred using a glass rod during extraction, which was carried out for 30 mins. The extracted muscle fragments were filtered through double folds of cheese cloth using a large funnel. The filtrate was saved for myosin. Gentle rocking of the cheese cloth speeded up the filtration. The residue, containing actin, was transferred to a 5 litre beaker.

4. Second extraction: The residue was suspended in 5 volumes of a freshly prepared solution of 0.4%  $\text{NaHCO}_3$ , 0.1mM  $\text{CaCl}_2$  made up in ice-cold glass distilled water. The contents were stirred continuously for

30 mins. Filtration was carried out as in the previous case using the same cheese cloth.

5. Third extraction: The residue was transferred to a 10 litre beaker and extracted for 10 mins with 1 volume of ice-cold 10mM  $\text{NaHCO}_3$ , 10mM  $\text{Na}_2\text{CO}_3$ , 0.1mM  $\text{CaCl}_2$  (freshly prepared) and stirred for 10 mins. Rapid dilution with 10 volumes of glass-distilled water (ice-cold) followed. Then the contents were filtered through the same cheese cloth used for the previous filtrations. The filtrate was discarded and the residue saved.

6. Acetone extraction: The residue was suspended at room temperature in 2 volumes of ice-cold acetone and stirred occasionally for 30 mins. The contents were filtered and the residue resuspended again in fresh ice-cold acetone. This procedure was repeated twice.

Finally, the dehydrated residue (acetone powder) was spread over a large filter paper sheet placed on a glass plate and allowed to dry overnight. The dried acetone powder was stored at  $-20^\circ\text{C}$ .

(b) *Purification of actin from acetone-powder:* The method of Kendrick-Jones, Lehman and Szent-Györgyi (1970) was followed to purify actin from acetone powder. Actin purified by this method is totally free of tropomyosin which occurs as a common contaminant in other methods (Rees and Young, 1967; Spudich and Watt, 1971).

1. Extraction of acetone powder with low ionic strength actin-extracting solution: A known quantity of acetone powder was extracted for 10 mins with 20 volumes of an ice-cold actin-extracting solution consisting of



0.1 mM ATP, 0.1 mM  $\text{Ca}^{2+}$  and 2 mM Tris pH 8.0. The contents were stirred occasionally using a glass rod. It is essential that the acetone powder is visibly swollen by the end of this extraction. The contents were filtered through the sintered glass funnel of a millipore filter using moderate suction. The residue was washed with the same extracting solution. The filtrate was clarified by spinning at 45,000 g for 1.5 hours.

2. Polymerization of actin: The actin which was present in the G-form in the supernatant was polymerized to the F-form by the addition of KCl and  $\text{MgCl}_2$  to bring their final concentrations to 10 mM and 0.7 mM respectively. This is a crucial step in the purification of actin because at this salt concentration only actin is polymerized and not tropomyosin. The polymerization was carried out first at room temperature for 30 mins and then at 4°C overnight. The polymerized actin was sedimented by spinning at 60-70,000 g for 4 hours at 4°C. The supernatant was removed and the tubes washed with a small quantity of 30 mM KCl, 10 mM Imidazole-HCl, pH 7.0, to remove excess ATP. The actin pellet was suspended in 30 mM KCl, 5 mM Imidazole-HCl pH 7.0 and gently homogenized with a teflon-coated pestle. The actin was used within 2-3 days. Thymol crystals were added to the F-actin solution to prevent bacterial growth.

All the procedures were carried out at 4°C, unless mentioned otherwise.

(c) *Isolation and purification of myosin:* Initial steps in the purification of myosin and the preparation of



acetone powder of actin are the same. The supernatant obtained after extraction of minced muscle with Guba-Straub's myosin-extracting solution was used for myosin purification and the residue was used for the preparation of acetone-powder of actin.

1. Myosin precipitation: The myosin-rich supernatant was clarified at low speed centrifugation ( $\sim 6,000$  g) at  $4^{\circ}\text{C}$  for 2 hours. The clarified supernatant solution was transferred to a large glass beaker and diluted ten-fold (in the cold room) with ice-cold glass distilled water, adding the water slowly around the sides of the beaker. This resulted in precipitation of myosin. Myosin was allowed to precipitate further in the cold room overnight.

2. Solubilization of myosin residue: The precipitated myosin was sedimented by low speed centrifugation ( $\sim 6,000$  g) at  $4^{\circ}\text{C}$  for 1 hour, and dissolved in a small quantity of 3M KCl by stirring. The dissolved myosin was then centrifuged at  $4^{\circ}\text{C}$  for 3 hours at 35,000 g.

3. Dialysis and further purification: The clarified myosin solution was dialysed against 50 volumes of 0.5M KCl and 5mM potassium phosphate buffer, pH 7.0, at  $4^{\circ}\text{C}$ . After overnight dialysis, the KCl concentration of the dialysis solution was reduced to 0.3M by dilution. Dialysis was continued for 5 more hours. The dialysed myosin was clarified at 35,000 g at  $4^{\circ}\text{C}$  for 24 hours.

After centrifugation, the concentration of KCl in the myosin supernatant was raised from 0.3M to 0.5M by the addition of solid KCl. An equal volume of ice-cold glycerol was then added to make the solution

50% (v/v). Then the stock myosin solution was stored in polythene containers at  $-15^{\circ}\text{C}$  in a freezer.

(d) *Preparation of heavy meromyosin:* Heavy meromyosin was prepared from myosin by tryptic digestion based on the method of Lowey *et al.* (1969). A portion of the stock myosin in 50% glycerol (for details of preparation of myosin, see p.39 in this chapter) was dialysed against 50 volumes of 30mM KCl, 10mM potassium phosphate buffer, pH 6.5, at  $4^{\circ}\text{C}$  overnight. The precipitated myosin was dissolved again by raising the ionic strength to 0.6M by the addition of solid KCl and the pH was brought to 7.0. Then, trypsin (TPCK) (253U/mg) (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) dissolved in 1mM HCl (0.05%) was added to the myosin solution till a ratio of 1:150 by weight between trypsin and myosin was achieved. The contents were stirred and the digestion was carried out at room temperature for exactly 10 mins. Digestion was stopped by the addition of soy bean trypsin inhibitor (type I-S) (Sigma Chemical Company, St. Louis, Mo, U.S.A.). The final concentration of the inhibitor was twice that of trypsin. The contents were stirred and dialysed against 60mM KCl and 10mM potassium phosphate buffer overnight at  $4^{\circ}\text{C}$ . The HMM fraction was clarified by centrifuging at 55,000 g for 2 hours. To the clarified supernatant of HMM an equal volume of glycerol was added and the HMM was stored at  $-20^{\circ}\text{C}$ .

(e) *Model experiments with actin and myosin:* Model experiments involving studies on the ultrastructure of purified rabbit skeletal muscle actin and myosin by themselves or a mixture of both were carried out to compare with the fine structure of cultured chick embryo connective tissue cells in order to identify the various cytoplasmic filaments and their associated structures in terms of their contractile protein nature. Purified F-actin filaments from skeletal muscle were also treated with HMM and DNAase I to compare with the results obtained from similar treatments of cytoplasmic filaments in the cells.

The procedures used for the isolation and purification of contractile proteins from skeletal muscle have been described earlier. Protein samples were processed for electron microscopy by either critical-point drying or negative staining as described below (see pages 52 & 53 in this chapter).

1. F-actin : Purified F-actin (see p.38 in this chapter) was dialysed against 50 volumes of 0.1mM ATP, 0.1mM  $\text{CaCl}_2$ , 10mM Imidazole-HCl, pH 7.0 at 4°C for 24 hours. During this process F-actin was depolymerized to G-actin. The G-actin was clarified at 70,000 g for 2 hours. G-actin was then polymerized by mixing equal volumes of a solution of G-actin and a solution of 0.1M KCl and 20mM Imidazole-HCl buffer, pH 7.0. The polymerization was carried out on the surface of a formvar/carbon-coated coverglass for 15 mins at room temperature. The polymerized F-actin was either critical-point dried (CPD) or negatively stained.

2.  $Mg^{2+}$  paracrystals of F-actin:  $Mg^{2+}$  paracrystals (Hanson, 1973) were formed by mixing equal volumes of F-actin or G-actin and a solution consisting of 50mM MgCl and 10mM Tris-HCl buffer pH 7.2. The paracrystals were immediately stained with uranyl acetate before examination with the electron microscope.

3. Myosin monomers and synthetic myosin thick filaments: A small portion of purified myosin stock solution (stored in 50% glycerol, 0.5M KCl, 10mM potassium phosphate buffer, pH 6.5, at  $-20^{\circ}C$ ) was dialysed overnight against 100 volumes of 0.1M KCl, 10mM potassium phosphate buffer, pH 7.0 at  $4^{\circ}C$ . This allowed the formation of myosin thick filaments. Samples of these thick filaments were either negatively stained or CPD.

To study the monomeric form of myosin, the stock solution of myosin was dialysed against 0.5M KCl, 10mM potassium phosphate buffer, pH 7.0 for 4-6 hours at  $4^{\circ}C$  to remove glycerol. From this dialysed myosin solution, samples were taken and processed for electron microscopy by critical-point drying.

4. F-actin-myosin monomer interaction: F-actin and monomers of myosin (obtained from the purified stock myosin solution) were mixed at a ratio of 4:1 by protein concentration. The mixture was allowed to stand at room temperature for 10 mins before fixation and critical-point drying.

5. HMM decoration of actin filaments: Purified HMM from rabbit skeletal muscle was stored as a stock solution in 50% glycerol, 60mM KCl, 10mM Tris-HCl, pH 6.5 at  $-20^{\circ}C$  (see p.41 ). This stock solution of



HMM was used either directly or after overnight dialysis against 60mM KCl, 10mM potassium phosphate buffer, pH 6.5 at 4°C to remove the glycerol. HMM and F-actin were thoroughly mixed in a test tube, at a ratio of 5:1 by protein concentration. After 30 secs, the actin/HMM mixture was diluted with 2-3 volumes of 60mM KCl, potassium phosphate buffer, pH 6.5. Then the actin-HMM complexes were examined by electron microscopy after negative staining. The  $Mg^{2+}$  paracrystals were also decorated with HMM and examined in the same manner.

6. DNAase I action on actin filaments: DNAase I prepared in standard salt solution (SSS) and F-actin were mixed at an equal molar ratio (3:4 by protein concentration) and mixed thoroughly. The mixture was kept at room temperature for 2 hours to examine the course of depolymerization of actin filaments by DNAase I. As a control for this experiment, to an equal amount of F-actin from the same preparation, SSS without any DNAase I was added. The test and the control were processed simultaneously for electron microscopy by negative staining.

$Mg^{2+}$  paracrystals of F-actin were also treated with DNAase I in the same manner as described above for F-actin. In some cases the treatment was extended up to 6 hours at room temperature. Controls using the same batch of  $Mg^{2+}$  paracrystals of F-actin were carried out as described in the controls for DNAase I: F-actin experiments.

#### Techniques Related to Chicken Gizzard Smooth Muscle

(a) *Isolation of intermediate filaments:* The procedure adopted here to isolate intermediate filaments is based



on the work of Small and Sobieszek (1977).

1. Isolation and cleaning of chicken gizzards:

For each experiment two chickens were sacrificed. The gizzards were removed immediately, wrapped in 'Glad wrap' and placed on ice. After 30-45 mins, when the gizzards were ice-cold, fat from the surface was removed by wiping with tissue paper. Each gizzard was cut into two halves and the contents were emptied. Using a blunt scalpel the tough layer of connective tissue was peeled from the gizzard. This leaves clean lateral smooth muscle.

2. Homogenization: Cleaned lateral muscles were

washed in ice-cold standard salt solutions (SSS) (Huxley, 1963) and coarsely minced with scissors. The minced muscle fragments were then vigorously homogenized in 10 volumes of SSS at high speed for 1.5 mins in 15 sec bursts with a 10 sec gap in between to avoid heating the muscle fragments. The entire process was carried out at 4°C. The homogenate was washed by centrifugation at low speed ( $\sim 2,500$  g) for 15 mins at 0-4°C. The residue was resuspended in SSS and centrifuged again. The supernatant solutions were discarded.

3. HAMES extractions: The washed homogenate was

extracted for 4 hours at 4°C with 5 volumes of high ionic strength acto-myosin extracting solution (HAMES) consisting of 0.6M KCl, 1.0 mM EDTA, 40mM Imidazole (pH 7.0), 5mM ATP, 1mM DTT (dithiothreitol) and 0.3% triton X-100. The extracted material was spun at 2,500 g for 15 mins. The residue was again extracted with 10 volumes of HAMES overnight at 4°C but with 25mM sodium pyrophosphate replacing the 5mM ATP. The residue was sedimented and

re-extracted with HAMES containing ATP and the cycle starting from 6 hourly extraction with HAMES and ATP was repeated until no more appreciable quantities of actin or myosin came out in the supernatant.

Finally the residue was suspended in HAMES, which did not contain either pyrophosphate, ATP or triton. From this suspension which was substantially enriched in intermediate filaments, samples were taken for electron microscopy.

(b) *DNAase I treatment of intermediate filaments:* Since DNAase I treatment of chick embryo connective tissue cells was carried out in SSS, the isolated intermediate filaments (which were suspended in HAMES) were dialysed against 10 volumes of standard salt solution (SSS) for 24 hours. Changes of dialysing medium were carried out every 8 hours.

A portion of the dialysed intermediate filament sample was treated with DNAase I at a final concentration of 2-5 mg/ml in SSS for 2 hours at room temperature and then overnight at 4°C. Another portion from the same batch of intermediate filaments without any added DNAase I was left standing at room temperature for 2 hours and at 4°C overnight as a control for DNAase I experiment. The control and DNAase I treated samples were both processed for electron microscopy in an identical way.

(c) *Trypsin treatment and HMM labelling of intermediate filaments:* The intermediate filaments were suspended in SSS as described above in the case of DNAase I treatment.

Then an attempt to label them with HMM as described for actin filaments (see p.43 ) was made. However, intermediate filaments did not label with HMM. Hence they were given a trypsin pre-treatment to see whether they would label with HMM as did the intermediate filaments of chick embryo connective tissue cells (see Chapter 4). For trypsin treatment, the residue which was rich in intermediate filaments, was dialysed against SSS as in the case of DNAase I treatment and suspended in an equal volume of trypsin (5 $\mu$ g/ml) in 50% glycerol/50% SSS. Trypsin was allowed to act on the intermediate filaments for 2 hours at room temperature. Immediately after trypsin treatment, the filaments were sedimented and resuspended in cold 50% glycerol/50% SSS. This was followed by several washings with cold SSS and finally the residue was negatively stained (see p.53) for viewing under the electron microscope.

#### Microscopical and Gel Electrophoretic Techniques

(a) *Light microscopy:* Light microscopy was used (i) to study the various movements exhibited by living cells, (ii) to select suitably well spread, healthy cultures for electron microscopy, and (iii) to gain an idea of how the cells respond to different extracting solutions (viz., change in shape, detachment from the surface, etc.).

The monolayer cell cultures in Rose chambers were examined with a Leitz Ortholux microscope fitted with Nikon high contrast phase contrast optics. The microscope was housed in an incubator box, maintained at

37°C with the aid of a thermostat-controlled warm air blower. The front of the incubator box had a removable panel of plexiglass to enable Rose chamber manipulation at the stage level and for focussing.

The Rose chamber, containing either normal or extracted cells, was mounted on the stage of the microscope. To obtain a clear view of the cells the formvar on the outer surface of the glass coverslip was removed, using a paper tissue soaked in glass-distilled water. The cells were examined under the low power and oil immersion. Photo micrographs were taken on Kodak (AHU) Microfile film using a Leica Camera with Mikas attachment for focussing. The film was processed in Kodak D19 developer for 5 mins.

(b) *Electron microscopy:* Processing of cells and protein samples for electron microscopic (EM) observations was carried out either by critical-point drying or negative staining.

1. Critical-point drying of cells: Normal cells and cells treated in various ways (e.g. glycerination, myosin extraction, HMM-labelling or DNAase I-treatment) were all processed for critical-point drying in a similar fashion. The only variation was that the treated cells underwent extensive washing with SSS before fixation while the normal cells were fixed immediately after removal from the Rose chamber.

i. Fixation: When the cells in a Rose chamber were ready for fixation, as indicated by light microscopic examination, the chamber was dismantled and its formvar or formvar/carbon-coated coverglass was quickly transferred,



cell side uppermost, into a 60mm diameter glass Petri dish containing 10ml of 0.8% (v/v) glutaraldehyde (Polysciences Inc., Warrington, Pennsylvania, U.S.A.), in 0.1M  $\text{CaCl}_2$ , 20mM Imidazole-HCl, pH 7.0. After 20 mins, the coverglass with attached cells was transferred to another 60mm diameter Petri dish containing 10ml of 4% glutaraldehyde in 0.1M  $\text{CaCl}_2$ , 20mM Imidazole-HCl, pH 7.0, for 4 hours. A few batches of glutaraldehyde were very acidic and, in those cases, the concentration of Imidazole-HCl was increased to 40mM. After glutaraldehyde fixation, the coverglass with attached cells was rinsed twice through Petri dishes of Hank's Balance Salt Solution (HBSS), pH 7.0. The coverglass was then transferred to an empty Petri dish and promptly covered with approximately 1 ml of 1% osmium tetroxide buffered with 0.2M sodium cacodylate (pH 6.5) for 10 mins. The coverglass with attached cells was washed twice through Petri dishes of glass-distilled water. Then the cells were further fixed by transferring the coverglass to an empty Petri dish and covering the attached cells with 1 ml of 1% (aqueous) uranyl acetate. After 10 mins the cells were again washed with glass-distilled water.

ii. Mounting of cells on EM grids: While still immersed within a Petri dish of glass-distilled water, the coverglass was observed with a dissecting microscope and a circular area of formvar film, to which cells were attached, was separated from the coverglass and turned over so as to bring the cells face down. Then, as soon as part of the formvar film reached the surface, the entire film promptly stretched out at the air-water interface.



Copper grids (H-I 200 mesh; H.F. Fullam Inc., Schenectady, N.Y., U.S.A.) were placed on suitably cellular areas of the floating formvar film. To make these grids adherent to the formvar, they were pre-coated by dipping them into a solution of 10% Milliners glue solution (Leighton, Melbourne, Victoria, Australia) and dried on filter paper. Alternatively, grids pre-heated to 55°C needed no glue to adhere to the formvar surface. After grids had adhered to the formvar, fine forceps were used to separate them with the cell-bearing formvar still attached. The base of a grid holder suitable for the critical-point drying apparatus (The Bomar Co., Tacoma, Washington, U.S.A.) was placed in the Petri dish (without disturbing the grids) and completely immersed under the water surface. Then the floating grids with attached formvar were secured (with the cell side uppermost) in the recesses of the grid holder base. The top of the grid holder was tightened firmly to the base, this preventing the grids from escaping while transferring the grid holder from one solution to the other and during the critical-point drying procedure.

iii. Dehydration: The fixed cells were dehydrated by transferring the grid holder through 15%, 30%, 40%, 50%, 60%, 70%, 80% and 90% acetone, each step lasting 1 min, then through 8 changes of 100% acetone, each for 2 mins.

iv. Critical-point drying procedure: Critical-point drying was carried out, using liquid carbon dioxide, in the Sorvall Critical-point Drying Apparatus (Ivan Sorvall Inc., Newtown, Connecticut, U.S.A.) as described

by Porter, Andrews and Kelley (1972). The critical-point drying apparatus (Fig. 3) consists of a pressure chamber with an inlet and an outlet controlled by pressure valves. The inlet is attached to a CO<sub>2</sub> cylinder and the outlet is attached to thickwalled silicone rubber tubing. A pressure gauge is also connected to the pressure chamber. The lid of the pressure chamber has a glass window to visualize the CO<sub>2</sub> bubbles which helps to regulate the rate of pressure increase during the initial stages.

Before critical-point drying cells, the pressure chamber was filled to three-quarters of its capacity with 100% acetone. After the final dehydration step the grid holder was transferred to the pressure chamber of the critical-point drying apparatus, the grids being completely covered with 100% acetone. The pressure chamber was closed tightly by screwing down the lid firmly to the base and closing both inlet and outlet valves. Then the inlet valve was opened gradually until it was observed that the CO<sub>2</sub> bubbled through the acetone in the pressure chamber at a rate of 1-2 bubbles/second. When the pressure reached  $\sim 800 \text{ kg/cm}^2$ , the inlet valve was opened fully. At this stage, the CO<sub>2</sub> pressure in the chamber became equal to the pressure in the CO<sub>2</sub> cylinder. Then the outlet valve was opened by a quarter of a turn to allow the liquid CO<sub>2</sub> to escape slowly. The valve was kept opened for 5 mins. This was followed by a 5 min period of closure of the outlet valve. This valve opening and closing procedure was repeated four times allowing all acetone to be replaced by liquid CO<sub>2</sub>. At the end of this process, both inlet and outlet valves

Fig. 3: Critical-point drying apparatus (for details,  
see p. 50, in this chapter)

C - pressure chamber.

I - liquid carbon-dioxide ( $\text{CO}_2$ ) inlet

O -  $\text{CO}_2$  outlet

W - water jacket.





were closed tightly and the pressure in the chamber was gradually raised to 1500-1600 kg/cm<sup>2</sup> by increasing the temperature in the chamber. This was achieved by partly immersing the pressure chamber in warm water at 55°C. When the pressure reached 1600 kg/cm<sup>2</sup>, the CO<sub>2</sub> outlet was opened by less than a quarter of a turn to release CO<sub>2</sub> very slowly. The decompression was gradual taking at least 10 mins. When the pressure reached zero, the outlet was opened completely, the grid holder was taken out from the chamber and grids were examined immediately. Grids required for further use were stored on dry filter paper in a Petri dish placed in a desiccator at 10<sup>-1</sup> Torr. The extra care taken to make only gradual pressure changes on the cells optimized fine structural preservation.

2. Critical-point drying of purified contractile proteins from-skeletal muscle: The protein was spread over a freshly glow-discharged formvar/carbon-coated glass coverslip (for preparation of formvar/carbon-coated coverslips see p.26). After 10 mins at room temperature (30 mins in the case of actin polymerization experiment), the coverslip was washed with several drops of standard salt solution (SSS). Instead of SSS, a solution containing 0.5M KCl, 10mM potassium phosphate buffer, pH 7.0 was used, in the case of critical-point drying of myosin monomers. After washing, fixation and critical-point drying was carried out as described previously for cells. However, in the case of F-actin, uranyl acetate fixation preceded osmium fixation. This reversal of order helped to preserve actin filaments, which are known to be highly susceptible to osmium.

3. Negative staining of cells: Negative staining was carried out according to the method of Huxley (1963). Unbuffered 1% or 2% aqueous uranyl acetate was preferred over other negative stains because of its known ability to preserve muscle filaments and to give high image contrast (Huxley, 1963). As reported by Huxley (1963) there was no sign that the preparations were adversely affected by the acidic pH (4.25 to 4.5) of the unbuffered uranyl acetate. Cultured cells which had been treated in various ways (e.g. with glycerol, HMM or DNAase I) were fixed by placing the coverglass with attached cells in 0.8% glutaraldehyde made up in the same salt solution used for that particular experiment. After 30 mins of fixation at room temperature, the coverglass was transferred to a Petri dish of distilled water and cell-bearing areas of carbon-coated formvar were transferred to EM grids as described in the critical-point drying procedure (see above). Several drops of 2% uranyl acetate solution were applied in quick succession to the grid. Excess negative stain was only partially removed with filter paper before the stain was rapidly dried in the hot airstream of a hair dryer. This drying procedure resulted in a more uniform thickness of negative stain and excellent image contrast.

4. Negative staining of proteins: Negative staining of proteins was carried out using carbon-coated EM grids.

i. Carbon-coating of EM grids: A thin layer ( $\sim 7.5$ nm thickness) of carbon was first coated onto a freshly cleaved fragment of mica using the same procedure

as described for carbon coating of coverslips (see p.26 in this chapter). The mica was slowly immersed in a Petri dish containing water, enabling the thin layer of carbon to float off from mica onto the surface of the water. While looking through a dissecting microscope, EM grids (pre-coated with glue) were applied to the thin layer of carbon. Using fine forceps the grids, with the attached carbon layer, were separated from the rest of the floating layer of carbon. These grids were then glow-discharged just before negative staining was carried out.

ii. Staining: A drop of protein solution was applied to a freshly glow-discharged grid. After 15 seconds, several drops of 2% aqueous uranyl acetate were applied to the grid. The subsequent procedure was the same as described for negative staining of cells.

5. Electron microscopic observation of processed specimens: The EM grids containing specimens processed either by critical-point drying or negative staining were examined under a Philips EM 301 microscope, fitted with a goniometer stage. An accelerating voltage of 80k.v. and an objective aperture of 70 $\mu$ m were used to view the specimen. Electron micrographs were taken using either a 35 mm camera or a plate camera. Stereoscopic pictures were taken by tilting the stage  $\pm 6^\circ$  from the horizontal. Instrument magnification was calibrated with a silicon monoxide grating replica having 2160 lines/mm.

(c) SDS polyacrylamide gel electrophoresis: Sodium dodecyl sulphate polyacrylamide (SDS) gel electrophoresis

was carried out to examine the various proteins present in normal cultured chick embryo connective tissue cells, glycerinated cells, myosin extracted cells and extracts obtained after glycerination, myosin extraction and DNAase I treatment. This technique was also applied to test the purity of proteins isolated from rabbit skeletal muscle and chicken gizzard smooth muscle. This was performed on slabs of uniform polyacrylamide concentration (either 5% or 10%) according to the method of Laemmli (1970) or on exponential polyacrylamide gradient gels as described by O'Farrell (1975).

1. Preparation of slab gels: Both uniform and gradient gels were prepared from a stock solution of acrylamide containing 30.0g acrylamide (Eastman Kodak Company, Rochester, N.Y., U.S.A.), 0.8g N,N'-methylene-bisacrylamide (Eastman Organic Chemicals, Rochester, N.Y., U.S.A.) and 0.1g sodium dodecyl sulphate (BDH Chemicals Ltd., Poole, England) in 100 ml deionized water. The solution was filtered through Whatman No. 1 filter paper to remove any insoluble material and stored in a glass-stoppered dark bottle at 4°C.

For uniform gels, the separating gel (5% or 10% acrylamide) was prepared in a beaker, on ice, by adding to the appropriate volume of stock acrylamide, SDS (0.1% (w/v) final concentration), separating gel buffer (0.375M Tris-HCl (pH 8.8) final concentration), N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma, St. Louis, M.O., U.S.A.) to a final concentration of 0.025% (v/v), and ammonium persulphate (BDH Chemicals, Poole, England) to a final concentration of 0.05% (w/v), in that order, and



mixed by swirling gently. Using a 10 ml pipette, the acrylamide mixture was then placed in the space between two 20cm x 20cm glass plates separated by perspex spacers about 1.5mm thick and sealed with yellow waterproof 'gel tape' (Universal Scientific Ltd., London, England). The plates were filled to within about 2.5cm of the top and subsequently gently overlaid with water using a 10 ml syringe and 25 G needle taking care to avoid mixing of the two layers.

For gradient gels, the polyacrylamide gradient was poured from a perspex two-chamber gradient mixer. The front chamber contained the high concentration of acrylamide and buffer prepared in 50% glycerol such that a density gradient could be formed in the gel. The back chamber contained the low concentration of acrylamide. The front chamber was stirred with a magnetic stirring bar which mixed in the low acrylamide solution as the gradient was being poured. The flow rate was about 2-3 ml/min. In order to form gradients of different concentration range, the proportions of acrylamide solution and water were varied appropriately. Each gel contained (final concentration) 0.375M Tris-HCl (pH 8.8), 0.1% SDS, 0.015% (w/v) ammonium persulphate and 0.05% (v/v) TEMED. All solutions were mixed in beakers in an ice bath before being added to the gradient mixer. The gels were overlaid with water as described for the preparation of uniform concentration gels.

After polymerization, about 1 hour for the uniform concentration gel and slightly longer for the gradient gels, the water overlay was tipped off and the top of

ADDENDUM

## Preparation of Cell Samples for Biochemical (electrophoretic) analysis

Cultured chick embryo connective tissue cells used for biochemical analysis involving SDS polyacrylamide gel electrophoresis were grown in T30 culture bottles [as contrasted to the cells grown in Rose Chambers for microscopical studies]. The culturing techniques such as preparation of culture medium, isolation of tissue, and separation of cells were the same as described earlier for cells grown in Rose Chambers (p. 26). To achieve a uniform distribution of cells in different culture bottles used for a given experiment, a constant amount of the same cell suspension was injected into each bottle which was found to give a well spread monolayer of cells.

The extraction of cells used for biochemical (electrophoretic) analysis was carried out as described for cells used for microscopical analysis (pp. 30-35). The cells were cultured for 24 hours and six bottles were used for each extraction procedure. Of the six, three were used as a control for that particular type of extraction.

To analyse different proteins in intact cells (cells which were not subjected to any type of extraction), T30 bottles were removed from the incubator after 24 hours of culturing and the culture medium from the bottles was decanted. The cells were dissolved by applying a solubilizing solution (1 ml/bottle) consisting of 0.0625M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5%  $\beta$ -mercaptoethanol and 0.04% bromophenol blue. To achieve complete solubilization, the contents were boiled for 1 min. The samples were used immediately for electrophoresis. The same procedure was also used to analyse proteins present in glycerol extracted, myosin extracted or DNAase I treated cells (pp. 30-35).

The extracts obtained after various extraction procedures were processed for biochemical analysis as follows:

The extract was spun at 2,500 g for 10 mins to sediment any cells that had floated off the glass surface of culture bottles during extraction. The supernatant from this centrifugation received trichloroacetic acid (TCA) to give a final concentration of 10% to precipitate protein. This was kept at 0°C for 1.5 hours. The precipitate was sedimented at 2,500 g for 10 mins and then washed once with ice-cold 10% TCA and once with ice-cold acetone before being solubilized in solubilizing solution as described above for cells. The contents were boiled for 1 min. The samples were used immediately for electrophoresis.

c) the gel was rinsed with additional water. Any remaining solution was subsequently removed by carefully blotting with a piece of filter paper. A perspex template of sample application wells was then placed above the separating gel.

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The ingredients for the stacking gel were also assembled on ice in a manner similar to that described for the separating gel. The stacking gel consisted of 3% or 5% acrylamide, 0.1% SDS, 0.125M Tris-HCl (pH 6.8), 0.05% TEMED, and 0.1% ammonium persulphate (final concentrations). The components were mixed by gently swirling and then added to the top of the separating gel taking care to remove any bubbles which may have formed. After polymerization (about 30 mins), the template was removed and the top of the gel was rinsed with water before overlaying with electrophoresis buffer which consisted of 0.005M Tris, 0.0384M glycine, 0.1% SDS (pH 8.3). In general, gels were stored overnight at 4°C before being used in order to insure complete polymerization of the acrylamide. This appeared to be particularly necessary for gradient gels to obtain optimal resolution.

2. Preparation of samples: Protein samples were prepared by completely dissociating in 0.0625M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5%  $\beta$ -mercaptoethanol, and 0.004% bromophenol blue as a tracking dye (final concentrations). Complete dissociation was brought about by immersing the samples in boiling water for 2-3 mins.

3. Electrophoresis: Samples containing up to

200mg protein (determined by the method of Lowry *et al.*, 1951) were loaded on the stacking gel beneath electrophoresis buffer using either a 5 $\mu$ l or 100 $\mu$ l glass syringe (Scientific Glass Engineering Pty. Ltd., Melbourne, Australia). Electrophoresis was performed at a constant current of 35 mA using power supplied by a b.w.d. model 216A stablized power supply (BWD Electronics Melbourne, Australia). After electrophoresis the gels were removed immediately and stained.

4. Staining and destaining: Gels were stained and destained using a modification of the method described by Fairbanks, Theodore and Wallach (1971). Gels were fixed and stained overnight at room temperature in 0.04% coomassie blue, 25% iso-propyl alcohol and 10% acetic acid. The staining solution was then removed and 25% iso-propyl alcohol, 10% acetic acid was added for 2-4 hours. This was followed by several changes of 10% acetic acid until the background was clear. Gels were then photographed.

5. Standards: A mixture of proteins of known molecular weights were co-electrophoresed with the test samples. A plot of relative mobility versus log molecular weight yielded a straight line from which an estimate of the molecular weights of the unknown proteins could be made (Figs. 4 and 5).

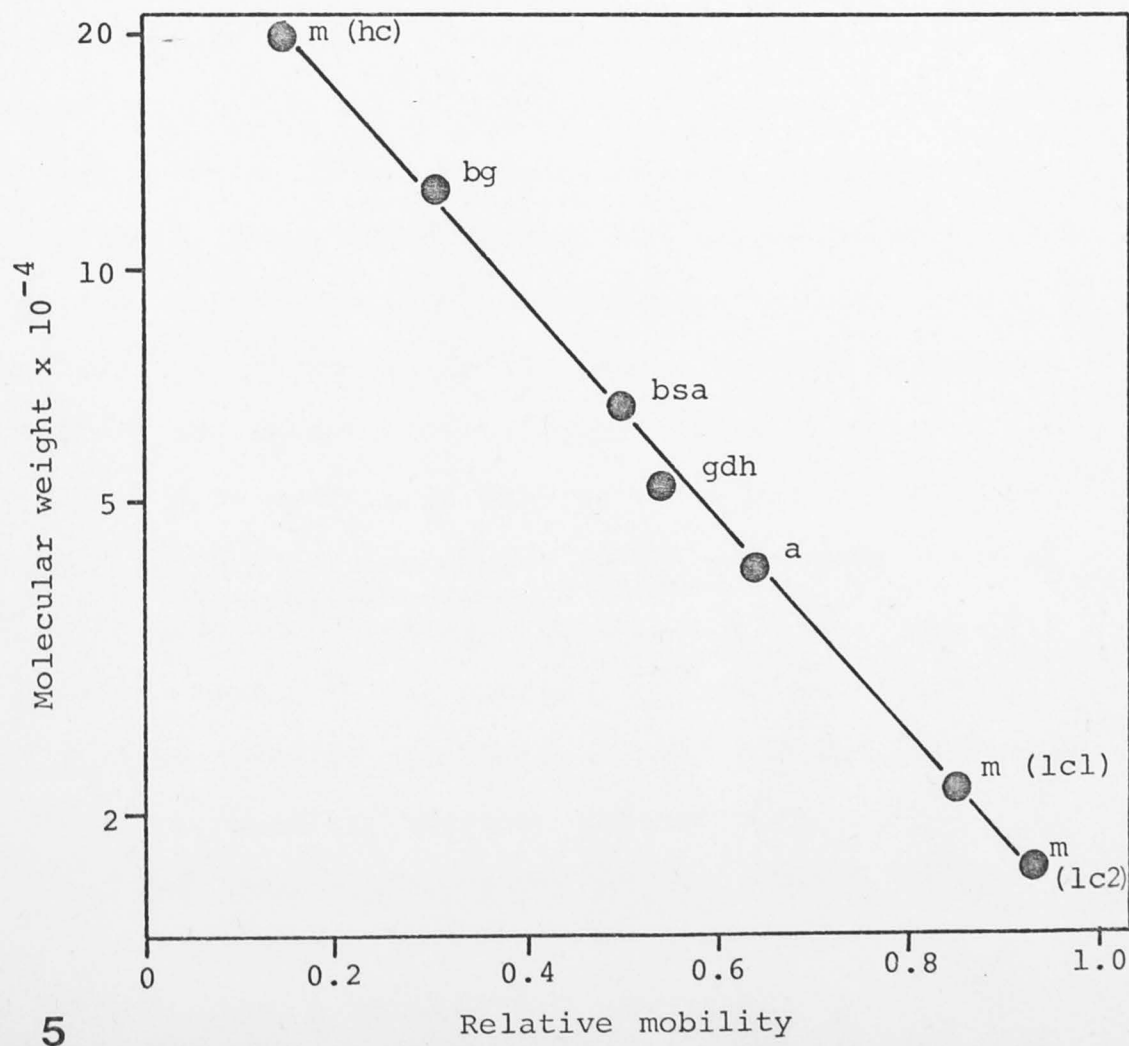
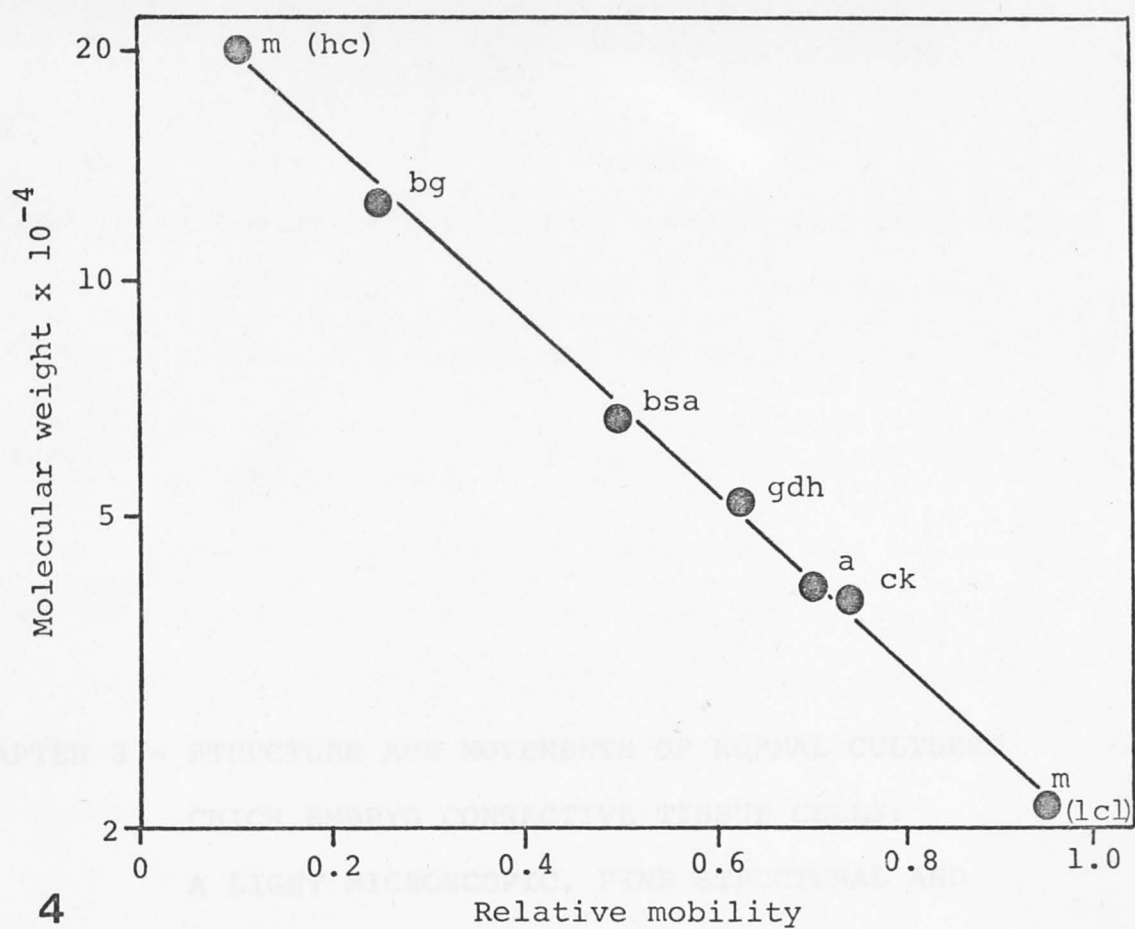


Fig. 4: Molecular weight standard curve obtained from a 10% gel as illustrated in Fig. 10, Chapter 7.

m (hc)	-	skeletal muscle myosin heavy chain
bg	-	$\beta$ -galactosidase
bsa	-	bovine serum albumin
gdh	-	glutamate dehydrogenase
a	-	skeletal muscle actin
ck	-	creatine kinase
m (lc1)	-	myosin light chain - 1.

Fig. 5: Molecular weight standard curve obtained from a 6½-15% gradient gel as illustrated in Fig. 12 in Chapter 6.

m (hc)	-	skeletal muscle myosin heavy chain
bg	-	$\beta$ -galactosidase
bsa	-	bovine serum albumin
gdh	-	glutamate dehydrogenase
a	-	skeletal muscle actin
m (lc1)	-	myosin light chain - 1
m (lc2)	-	myosin light chain - 2



CHAPTER 3 - STRUCTURE AND MOVEMENTS OF NORMAL CULTURED  
CHICK EMBRYO CONNECTIVE TISSUE CELLS:  
A LIGHT MICROSCOPIC, FINE STRUCTURAL AND  
BIOCHEMICAL STUDY

## INTRODUCTION

The various movements exhibited by cultured chick embryo connective tissue cells of the fibroblast type have been established by light microscopy and cine photomicrography (reviewed by Buckley, 1964). Two broad categories of subcellular motility occur. These are: deformational movements of the cell surface (seen especially at the cell margin) and extended migrations of organelles through the sub-cortical cytoplasm (Buckley, 1974). Before attempting to postulate mechanisms for the subcellular movements, it seems important to define details of the fine structure associated with the motile regions of the cell.

Using sectioned cultured cells, many workers have found within the motile margins of these cells, that there are always masses of fine sub-plasmalemmal filaments (e.g. Spooner, Yamada and Wessels, 1971; Wessels, Spooner and Luduena, 1973; Goldman, Chang and Williams, 1975). Similarly, although the association has seemed less constant, within the sub-cortical cytoplasm, motile organelles are surrounded by filaments and microtubules (Porter, 1976). These associations have encouraged many investigators to propose that filaments are in some manner involved in both forms of subcellular movements (Buckley, 1974). As mentioned in Chapter 1, the presence of both actin and myosin in many types of non-muscle cells, including cultured connective tissue cells, has been demonstrated using biochemical and immunofluorescence methods (Hatano and Oosawa, 1966; Adelstein *et al.*, 1972; Lazarides, 1976;



Weber and Gröeschel-Stewart, 1974).

If movement in non-muscle cells is brought about by actin-myosin interactions, as in skeletal muscle cells (Pollard and Weihing, 1974), the pronounced dissimilarity in form and arrangement of the filaments in non-muscle cells such as cultured connective tissue cells (cf. skeletal muscle cells) has made it seem difficult to explain how an actin-myosin system comparable to that of skeletal muscle could function in such cells. However, it seems relevant to point out that the movements exhibited by cultured chick embryo connective tissue cells differ significantly from those of skeletal muscle cells, both in their character and in their complexity (Buckley and Raju, 1976). With this in mind, it may not be reasonable to expect that actin and myosin should have a muscle-like arrangement in such non-muscle cells. Accordingly, there appears to be a need to gather more detailed information concerning the fine structural organization of the cytoplasmic filaments of non-muscle cells, and, if possible, to designate the protein identity to these filaments.

The fine structural details obtainable from studies based on thin sectioned material are not adequate because there are often some doubts about the form and dimensions of individual filaments and, more importantly, many doubts about their three dimensional distribution in relation to one another and to the cells' moving parts.

An alternative approach to elucidating the three dimensional distribution of filaments, microtubules and other components within cultured chick embryo cells is based on processing whole cells for electron microscopy by

fixation and then critical point drying through liquid carbon dioxide (Anderson, 1951; Buckley and Porter, 1973). By examining stereoscopic pairs of electron micrographs of cells prepared in this way, the three dimensional disposition of organelles, cell margins and cytoplasmic matrix components in relation to one another can be meaningfully evaluated. This method was successfully applied by Buckley (1975), Buckley and Porter (1975) and Buckley and Raju (1976).

A further reason for this work is that a clear understanding of the structure of normal cells with respect to the arrangement of their cytoplasmic filaments is essential for interpreting the results obtained with cells extracted for particular contractile proteins, the results of which will be described in the following chapters. The present studies describe the structure of normal cultured chick embryo connective tissue cells under light microscopy using phase contrast optics. Then their fine structure, using improved methods of fixation and critical-point drying, is described. Light microscopy was carried out to gain an appreciation of the complex movements of living cells and to demonstrate that the fixation and critical-point drying used in these procedures do not alter the shape of the cell or the normal intracellular arrangement of organelles.

The results presented in this chapter are grouped together as follows:

and the details regarding the cell margins can be readily appreciated. The cytoplasmic nucleus is often centrally located within the cell. Nucleoli are phase

Light (Phase Contrast) Microscopy of Living CellsElectron Microscopy

- (a) General description of the fine structure of fixed, critical point dried (CPD) cells;
- (b) Description of the fine structure of filament systems of cells;
- (c) Histogram showing the diameter distribution of the various filaments.

SDS Polyacrylamide Gel Electrophoresis of Normal CellsRESULTSLight (Phase Contrast) Microscopy of Living Cells

As illustrated in the low power photomicrograph (Fig. 1), cultured chick embryo connective tissue cells are thinly spread out polyhedral structures, sometimes with many polar projections. However, the precise shape of individual cells varies from one cell to another. The cell margin is frequently extended into long processes and in some areas, the margin shows undulations. An ovoid nucleus, nucleoli and particulate organelles are visible even at low power. However, precise details of the structures of the living cell cannot be appreciated at such low magnification.

When the cells are viewed at high magnification (Figs. 2 and 3), the structure and disposition of various organelles and the details regarding the cell margin can be more readily appreciated. The ovoid nucleus is often eccentrically located within the cell. Nucleoli are phase

Fig. 1: Low power phase-contrast micrograph showing one day-old cultured chick embryo connective tissue cells of fibroblastic type. The cells are thinly spread out. Cell margins (cm) are gently curving and many are extended into long processes. In some areas where the cell margin is expanded, it appears to be undulating (arrows). Nuclei (n) are mostly ovoid and nucleoli (nc) can be easily identified. In many cells there are phase-dark particles (p) suggesting the presence of particulate organelles. X 560.



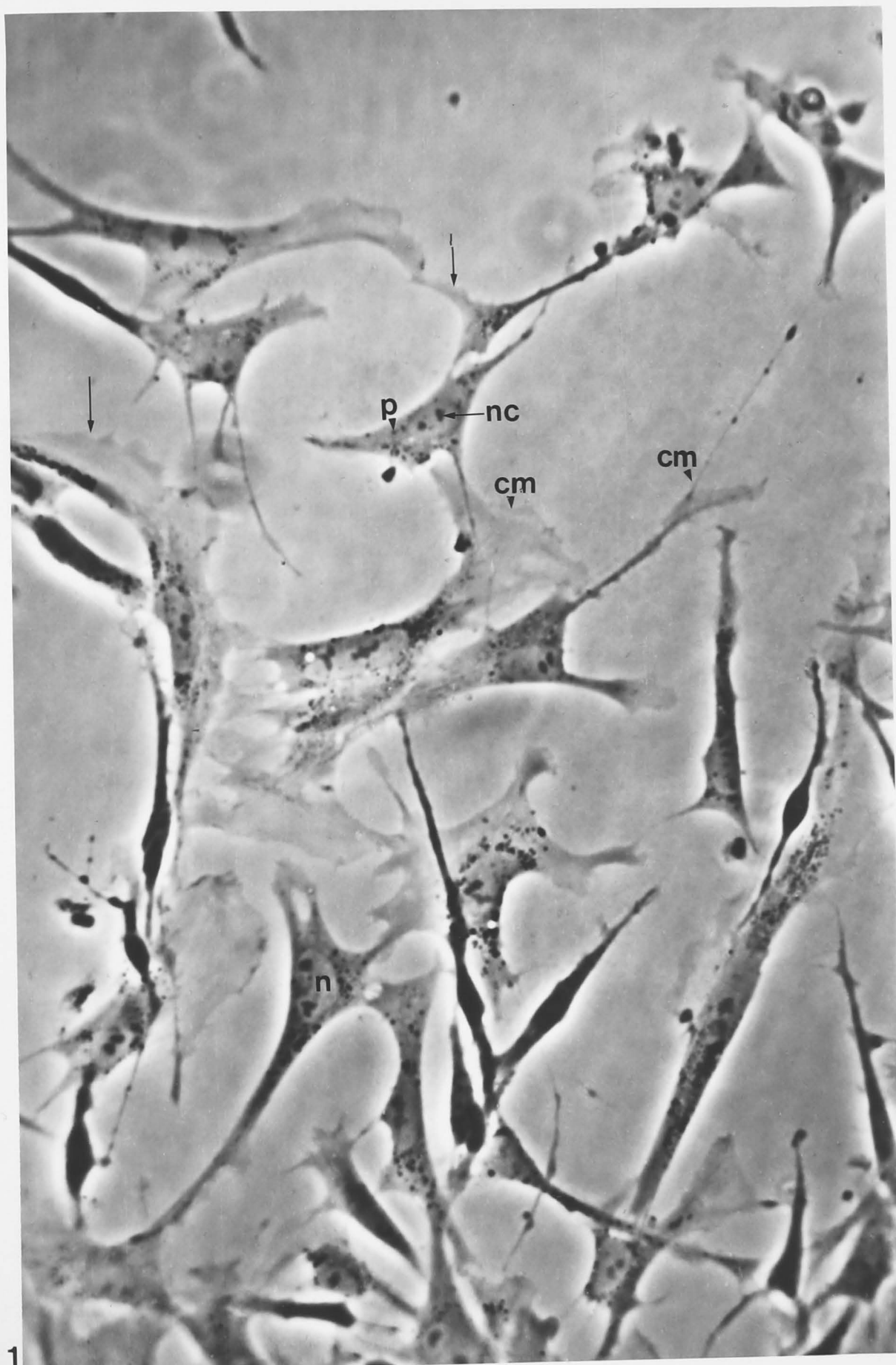


Fig. 2:Phase-contrast micrograph of a living cell taken using oil immersion objective showing the eccentrically located nucleus (n), phase-dark nucleoli (nc), mitochondria (m), lysosomes (ly), Golgi apparatus (g) and endoplasmic reticulum (er). The cell margin is gently curving and in some areas it is drawn into veil-like extensions (v). From these veils finger-like microvilli (mv) project out into the growth medium. X 2100

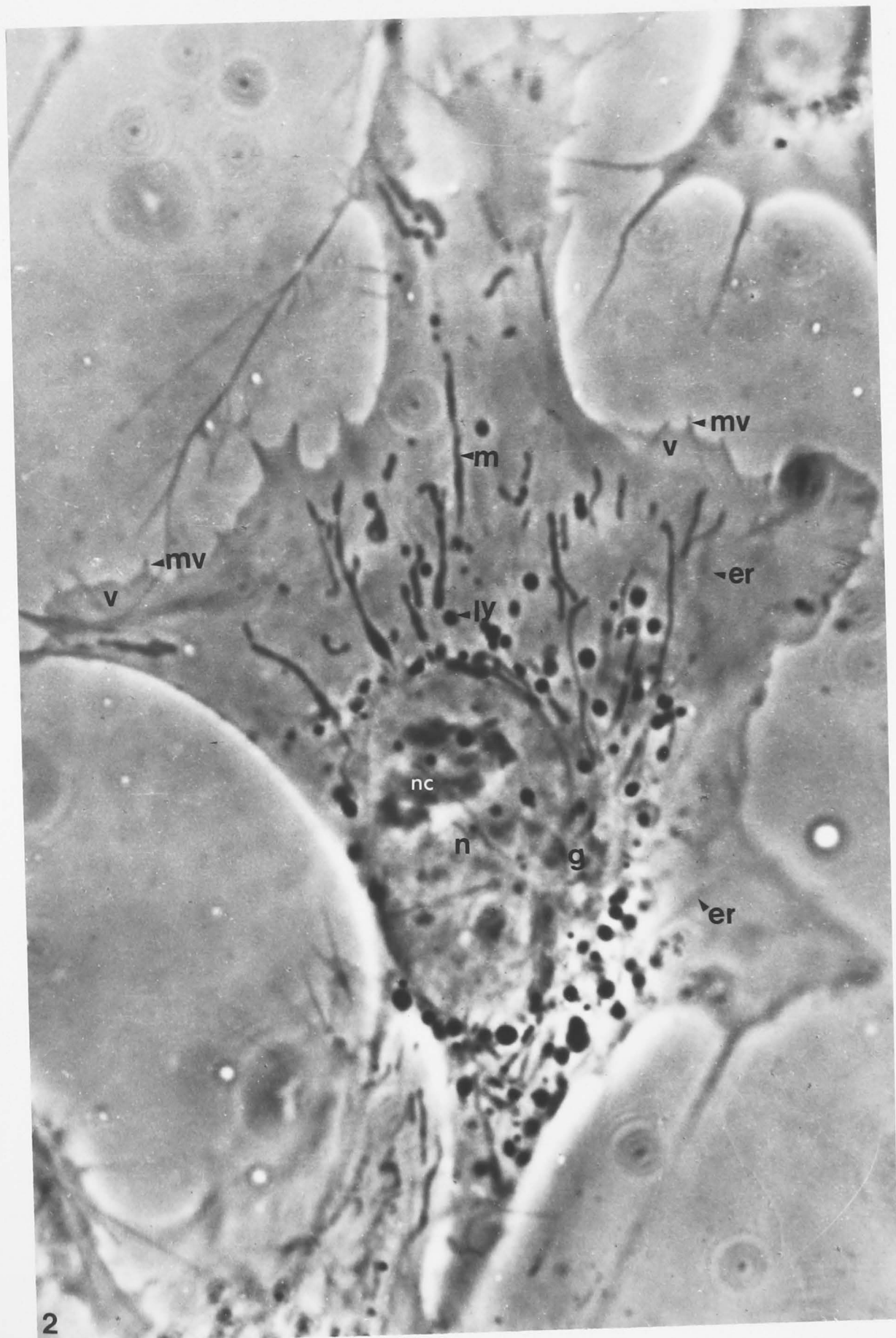


Fig. 3: Phase contrast micrograph of a cell similar to that shown in Fig. 2, illustrating long and straight stress fibres (sf), in addition to the features described in Fig. 2. Refractile lipid bodies (l), which are not clearly distinguishable in Fig. 2 can be easily identified in this micrograph. X 1400





dark in appearance and highly variable in shape. Situated in the perinuclear region, is the Golgi Complex. This is a crescent-shaped structure which is largely free of other organelles. Mitochondria are identified as long, thread-like, phase dark structures which may be straight or undulating, occasional ones showing branching. Lysosomes are phase dark, round or ovoid structures. Lipid bodies appear as round, phase light structures with phase dark margins.

These particulate organelles are concentrated about the nucleus. Because of its extremely low contrast, endoplasmic reticulum is difficult to resolve. However, faint, phase dark strands probably represent portions of the endoplasmic reticulum scattered throughout the cytoplasm.

Over much of their extent, the margins of these polygonal cells appear sharply defined and gently curving. However, in various places, particularly at the projecting poles of the cell, the cell margin is thrown out into thin, veil-like extensions shaped more or less like a fan (Figs. 2 and 3). Commonly from these, narrow finger-like processes termed microvilli can be seen projecting out (Figs. 2 and 3). In addition, at the base of the veils and elsewhere throughout the cytoplasm are phase dark striations known as 'stress fibres' or 'tension striae' (Lewis and Lewis, 1924) (Fig. 3). From both light and electron microscopic studies, it is known that these are located within the cell cortices (Buckley and Porter, 1967). Their number and distribution vary from cell to cell.

Except for the Golgi complexes, lipid inclusions and stress fibres, the cytoplasmic components, exhibit a constant

motion which can be seen either directly or more effectively by reviewing time-lapse movie records. The movement of particulate organelles which migrate swiftly through the cytoplasm on seemingly straight paths is rather striking. Periodically such moving organelles stop and, after a brief pause, start moving again in some new direction. The direction and speed of movement of individual organelles appear to be independent of each other.

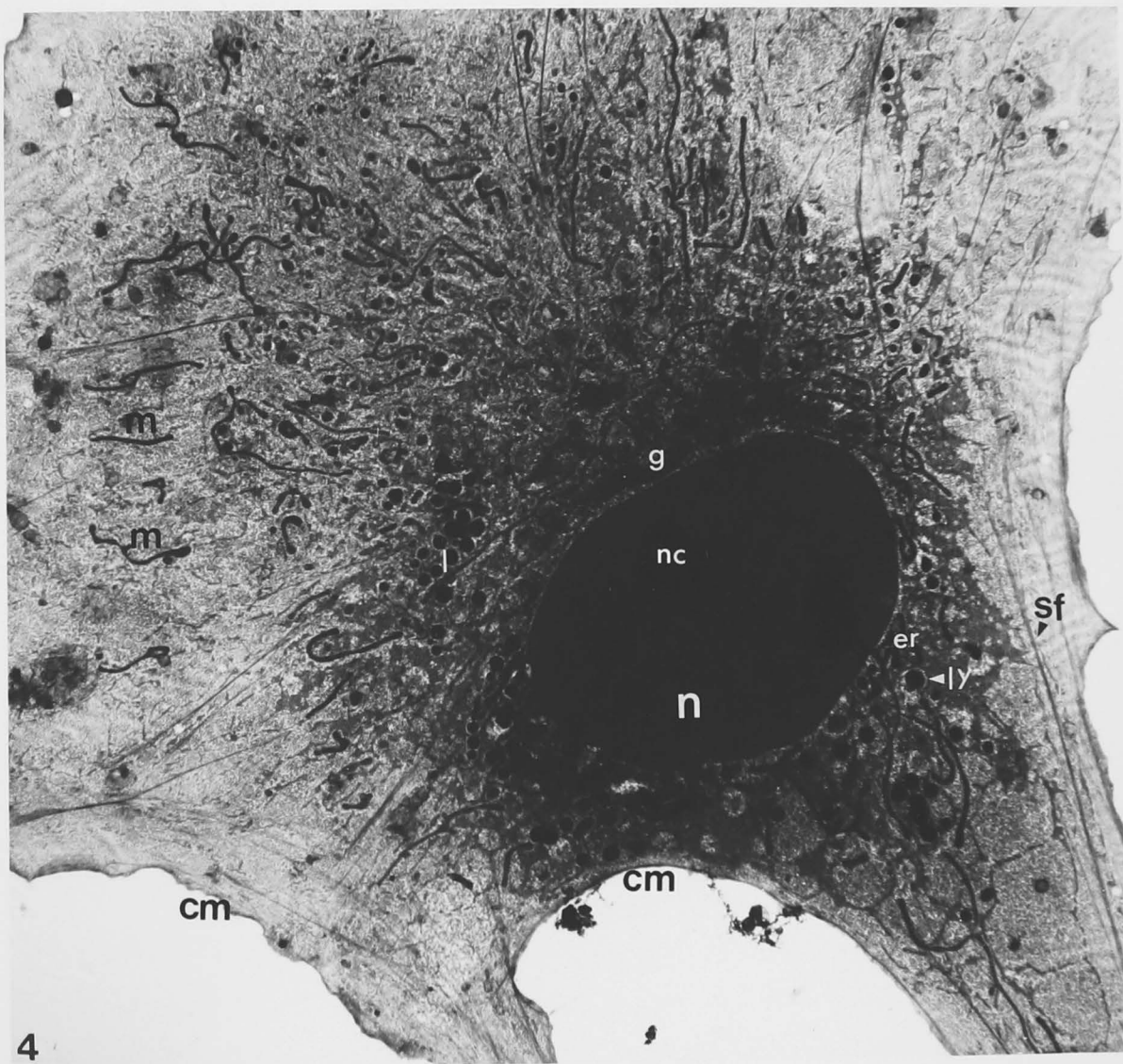
Equally striking are the movements shown by the veil-like extensions of the cell margin and the microvilli. Continually, the veils exhibit highly complex undulatory movements, extensions and retractions. In some instances, these retractions are accompanied by pinocytosis (i.e. the uptake of droplets of liquid from the culture medium). Microvilli, which extend out from the veils, commonly undergo a series of bending movements before disappearing by being retracted back into the veils.

#### Electron Microscopy

(a) *General description of the fine structure of fixed CPD cells:* The structure of the CPD cell, as seen at low magnification (Fig. 4) bears a striking similarity to that of the living cell seen with the phase contrast microscope (Fig. 2). As illustrated in Fig 4, an eccentrically located, ovoid nucleus contains two electron-dense nucleoli. The Golgi complex is located adjacent to the nucleus on one side. Elsewhere, close to the nucleus, because of the thickness of the cytoplasm, organelles appear at high density. Nevertheless, one can readily identify thread-like mitochondria of varying lengths, lysosomes, lipid droplets and cisternae of the endoplasmic reticulum. Here, close

Fig. 4: Low power electron micrograph of a fixed, CPD cell (for methods of preparation, see Chapter 2, p.50 ). This cell shows features which can be readily correlated with phase-contrast micrographs of living cells (cf. Figs. 2 and 3). An ovoid nucleus (n) is eccentrically located and nucleoli (nc) are highly electron dense. The Golgi apparatus (g) is crescent-shaped. Organelles appear concentrated about the nucleus because of the thickness of cytoplasm in that region. Nevertheless mitochondria (m), lysosomes (ly), lipid bodies (l) and endoplasmic reticulum (er) can be readily identified. Although it is hard to distinguish between lysosomes (ly) and lipid bodies (l) (both are electron dense and spherical or ovoid in shape) whereas lysosomes are dispersed randomly, lipid bodies are arranged into more or less regular clusters. The endoplasmic reticulum (er) appears as broadly fenestrated and cisternae are located around the nucleus. Peripherally, these cisternae give off finer strands. Straight or slightly curving stress fibres (sf) can be seen radiating throughout the cytoplasm. The cell margin (cm) is undulating in some areas and gently curving in others. X 3,700.

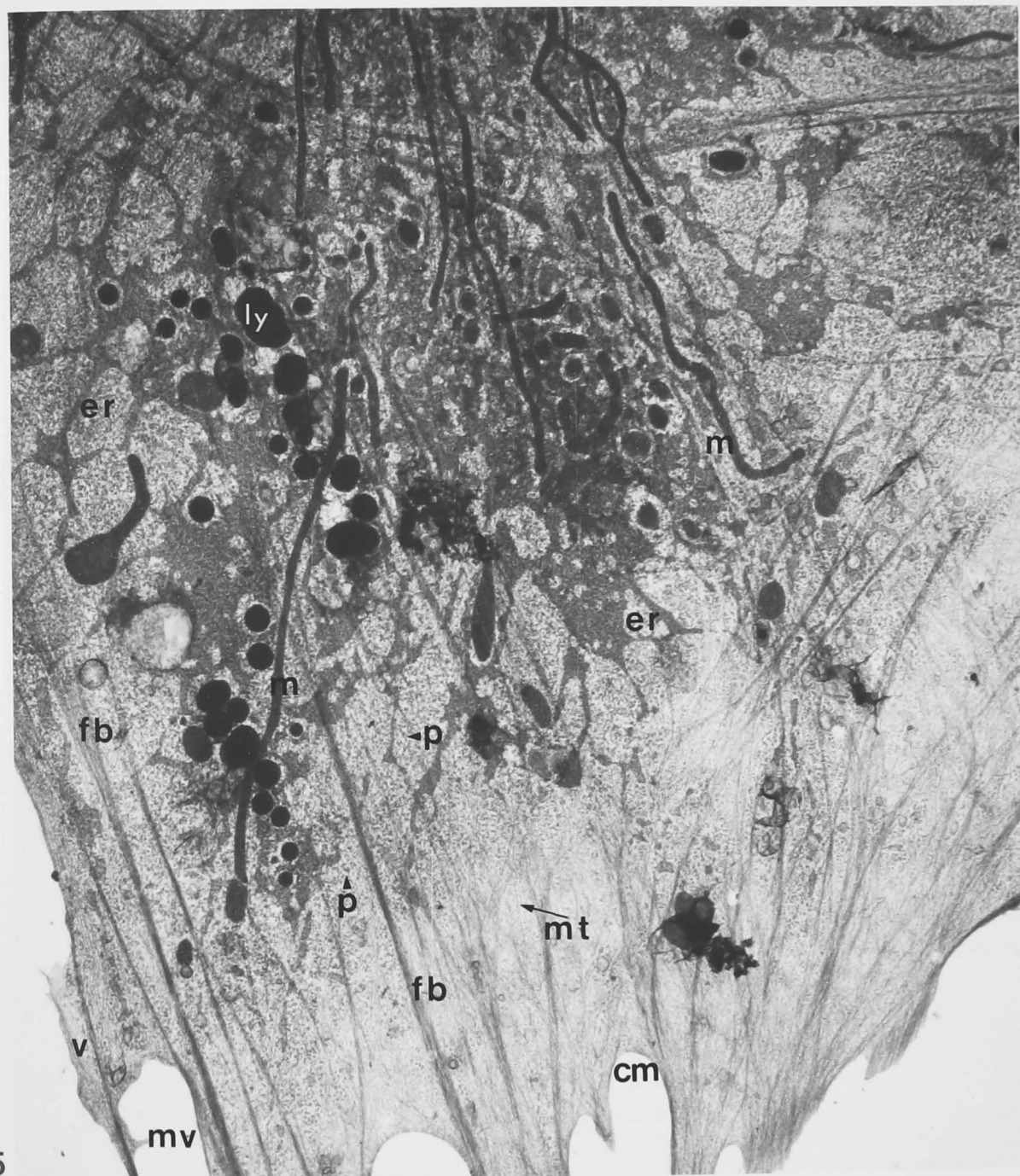




to the nucleus, the endoplasmic reticulum appears in the form of broad fenestrated cisternae which, peripherally, give off the finer strands that form the more dispersed reticulum. Long straight or gently curving stress fibres can be seen radiating throughout the cytoplasm. The cell margin appears undulating in some areas and gently curving in others.

With slightly higher magnification (Fig. 5), it can be seen that the stress fibres are composed of bundles of filaments. It is not possible to resolve individual filaments distinctly. However, it is clear that whereas some fibres remain as fairly tightly packed bundles of more or less uniform calibre, many others fan out to form more diffuse filament arrays. The microtubules are long and straight or slightly curving structures of uniform calibre ( $\sim 19\text{nm}$ ). These traverse the cytoplasm in various directions. The reticular pattern of both the fenestrated cisternae and more disperse forms of the endoplasmic reticulum can also be appreciated in this electron micrograph (Fig. 5). Various particulate organelles, mitochondria and lysosomes can be seen in close association with the more compact forms of endoplasmic reticulum. Included in the cytoplasm are numerous tiny electron-dense granules which, by comparison with sectioned cells, can be identified as polyribosomes. The cell margin is scalloped and, in a few areas, it has formed veil-like extensions. This is more clearly demonstrated in the following stereo pair of micrographs. Figs. 6a and b show the pole of a cell containing filament bundles, microtubules, coated vesicles and numerous

Fig. 5: Electron micrograph showing a portion of a CPD cell under slightly higher magnification than in Fig. 4. It can be seen that the structures described as stress fibres in Figs. 3 and 4 are in fact composed of bundles of filaments (fb). It is not possible to resolve individual filaments distinctly. The length as well as the width of filament bundles vary considerably. Whereas the filaments are tightly packed in some bundles, in other bundles the filaments fan out to form diffuse arrays. Elongate microtubules (mt) can be seen traversing throughout the cytoplasm. The reticular pattern of the fenestrated endoplasmic reticulum (er), mitochondria (m), lysosomes (ly) and numerous electron dense particles which, by comparison with sectioned cells, can be identified as polyribosomes (p) are also illustrated in this micrograph. The cell margin (cm) appears scalloped and in some areas extended into veil-like (v) extensions from which microvilli (mv) project out.  
X 7,000.

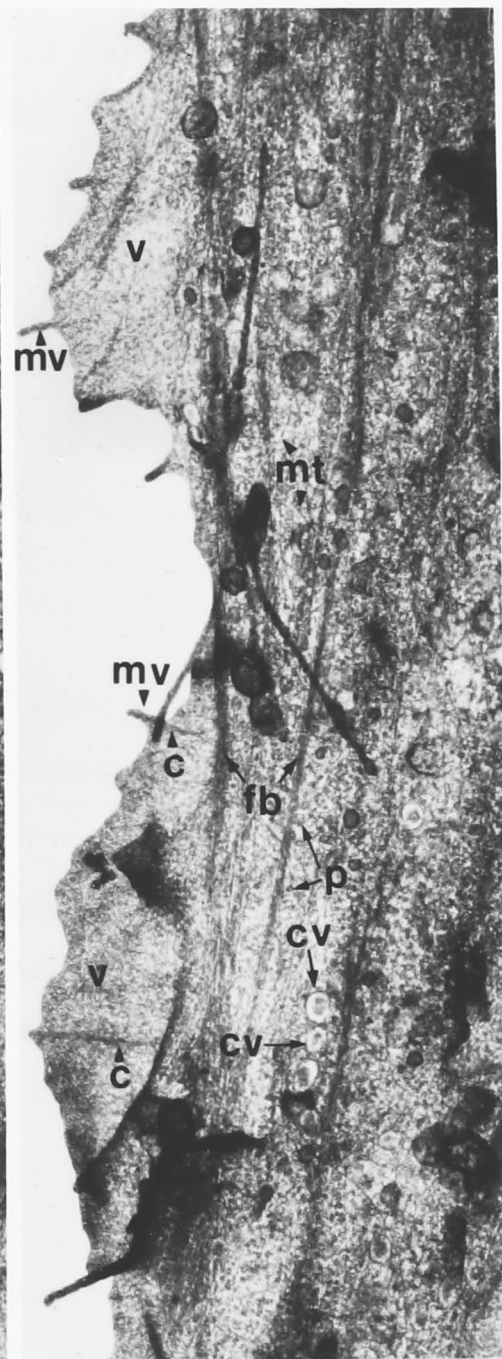




Figs. 6a and b: A stereo pair of electron micrographs illustrating the features of veil-like extensions (v) of the cell margin. These veils in general project upwards from the surface. Finger-like microvilli (mv) extend out from the veils. Within each microvillus is a core element (c) consisting of closely aligned filaments which extend deep into the base of the veil. The rest of the structures within the veils appear as a highly complex meshwork and at this magnification it is not possible to resolve further details. Filament bundles (fb), microtubules (mt), coated vesicles (cv) and polyribosomes (p) can be also seen.  
X 15,400



6a



6b

polyribosomes. The cell margin is extended into a series of fan-like extensions or veils, which are very similar to the ones described earlier in the phase contrast images of living cells. Microvilli can be seen projecting out from these fan-like extensions. These microvilli contain a dense core of filaments which project down towards the base of the veil. Elsewhere within the veils, filaments occur in less regular arrays, appearing as a feltwork.

The overall complexity of the filament systems, both within these surface projections and deeper within the cell is remarkable. This aspect of the cell's fine structure, with which the present study is mostly concerned, will now be described in detail.

*(b) Description of the fine structure of filament systems of cells:* The filament systems comprise filaments ranging in diameter from 4 to 11 nm. Based on their size, the filaments of non-muscle cells have been classified as 'thin' and 'intermediate' by Ishikawa, Bischoff and Holtzer (1968). In the present study 4 to 7 nm diameter filaments will be regarded as 'thin' filaments and 7 to 11 nm diameter filaments as 'intermediate' filaments.

The most obvious and striking system of filaments are those which make up the stress fibres, the fibrous structures which consist of bundles of closely parallel thin filaments (Figs. 6a and b). These filaments are long, relatively straight, and often tightly packed. However, not uncommonly the filaments fan out from their tight

bundles to form sheet-like arrays. This is a very common occurrence where the fibres approach their terminations towards the cell margins. The width and the length of the bundles vary, though frequently they extend across the entire length of the cell, within both dorsal and ventral cortices.

In the sub-plasmalemmal cytoplasm of the veils thin filaments predominate (Fig. 7). Here they form a very fine meshwork, cross-linked by short structures which are described below. Commonly the filaments are almost totally obscured by these cross-linking structures. The filamentous core which occurs within microvilli, is seen as a narrow bundle of more or less parallel but loosely allied thin filaments. These filamentous cores extend towards and sometimes penetrate the cortical bundles. In these veil-like sub-plasmalemmal regions, microtubules are seen only on rare occasions.

The cortical bundles in the dorsal and ventral regions are interconnected by a complex three dimensional network of filaments which appear to extend throughout the entire sub-cortical cytoplasm. The features of this sub-cortical network of filaments are illustrated in Figs. 8, 9a, b and 10a and b. Included in the sub-cortical filament system are both thin and intermediate filaments which branch and anastomose with one another to give the network a three dimensional character. The great majority ( $\sim 90\%$ ) of these sub-cortical filaments are intermediate in size and take long meandering courses, branching as they go.



Fig. 7: Electron micrograph of a portion of a fixed, CPD cell showing details of the sub-plasmalemmal cytoplasm within a veil (v). A microvillus (mv) can be seen projecting out from the veil. The cytoplasm within the veils contains many 4-7 nm diameter filaments (f). However, these are difficult to discern clearly as they are largely obscured by numerous cross-linking structures which are mostly 'dumb-bell' shaped (arrows). Because of the extensive cross-linking, a fine meshwork is formed. At the base of the veil, there is a filament bundle (fb) consisting of closely aligned, parallel thin filaments. Microtubules (mt) can be seen in the deeper cytoplasm. X 54,600.

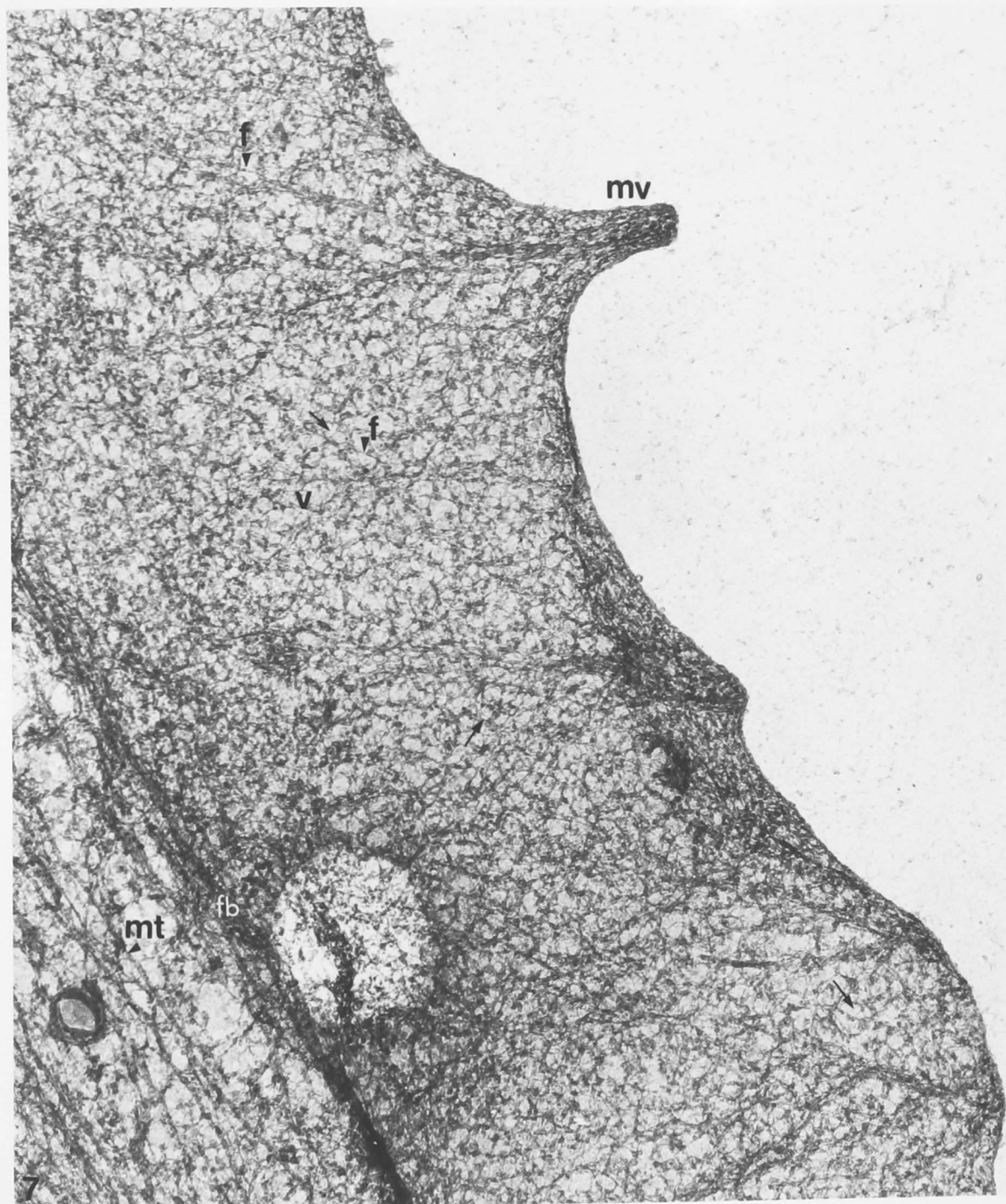
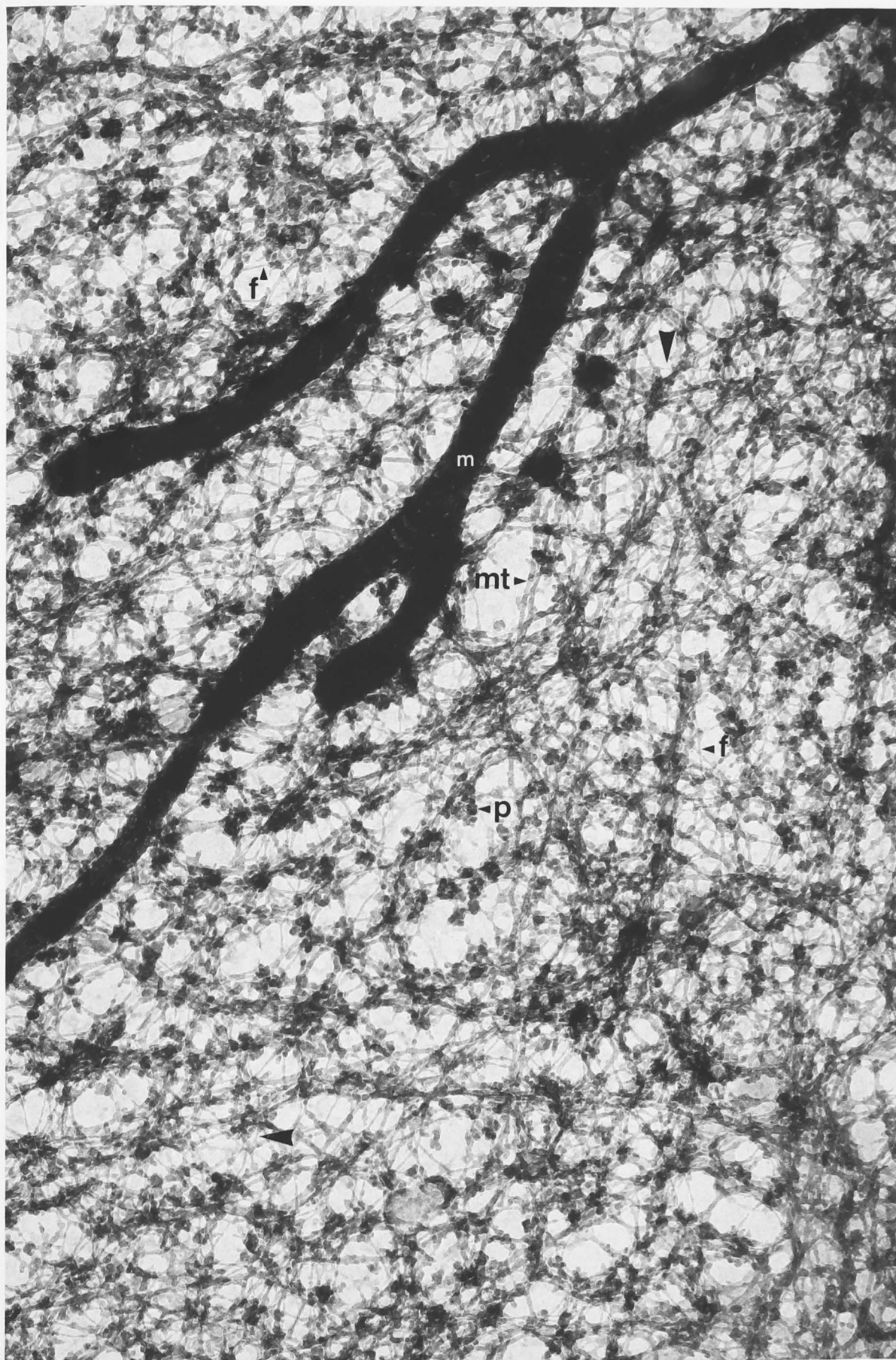


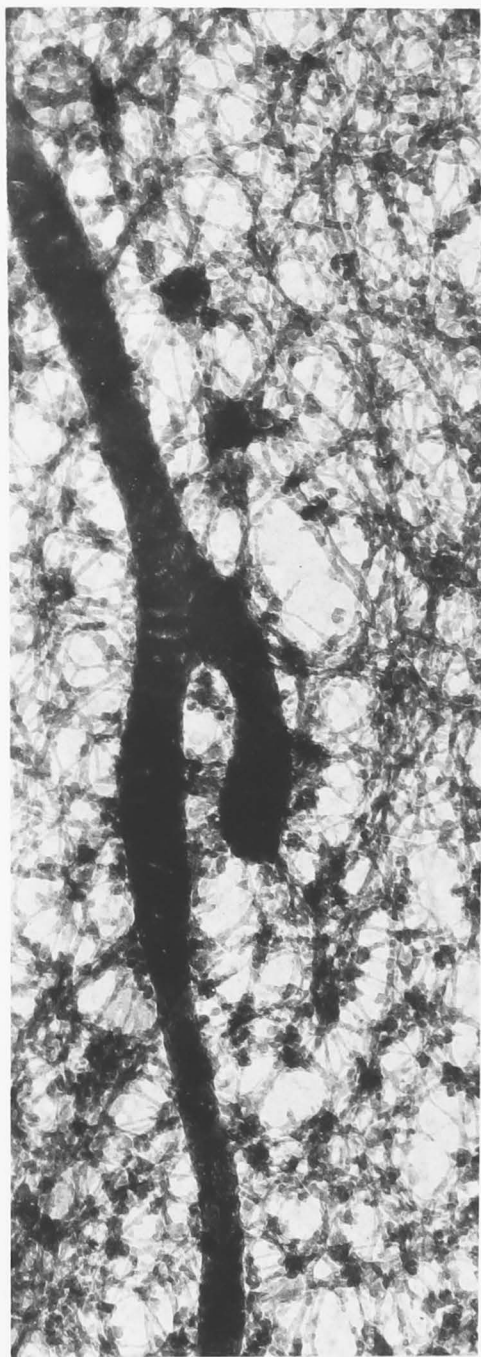
Fig. 8: This electron micrograph shows the features of the sub-cortical cytoplasm of a CPD cell. A long branching mitochondrion (m) with fairly well-defined crystae can be seen. This mitochondrion is surrounded by a network of filaments (f) consisting of 4-7 nm diameter thin filaments and 7-11 nm diameter intermediate filaments. Microtubules (mt) also take part in this network and are widely distributed throughout the cytoplasm. There are also clusters of electron-dense polyribosomes (p). Cross linking these to the abovementioned filaments are some short cross-linking elements appearing as nodes with fine tails (arrowheads). X 52,000.



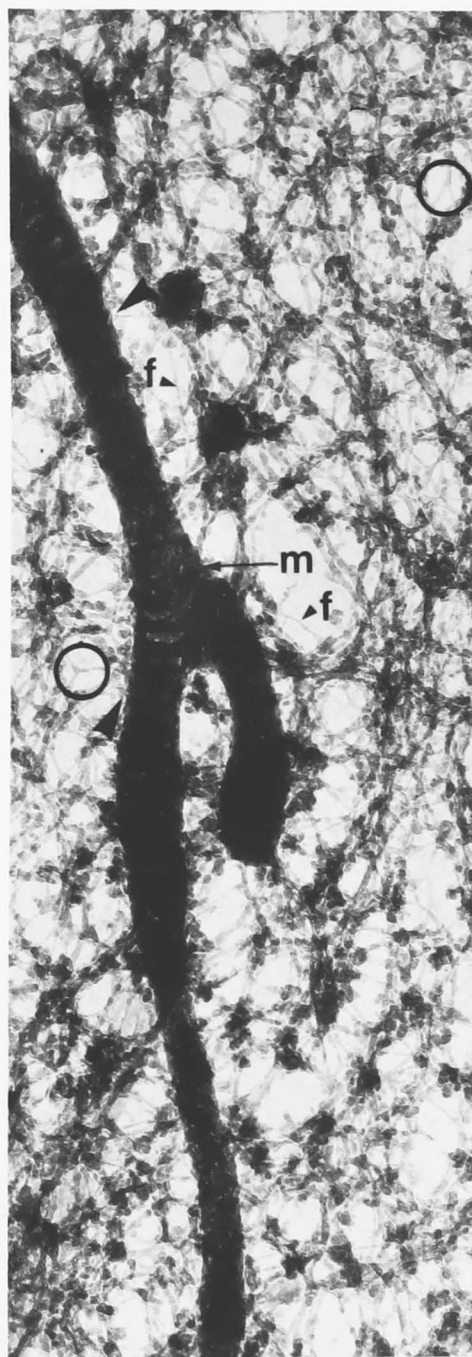




Figs. 9a and b: Stereoscopic illustrations of a portion of the cell shown in Fig.8. The mitochondrion (m) is surrounded on all sides by a three dimensional network of filaments (f). Branching of filaments (circles) is clearly seen in some areas. A direct link between the mitochondrion and the filament network is highly suggestive (large arrowheads). X 53,500.

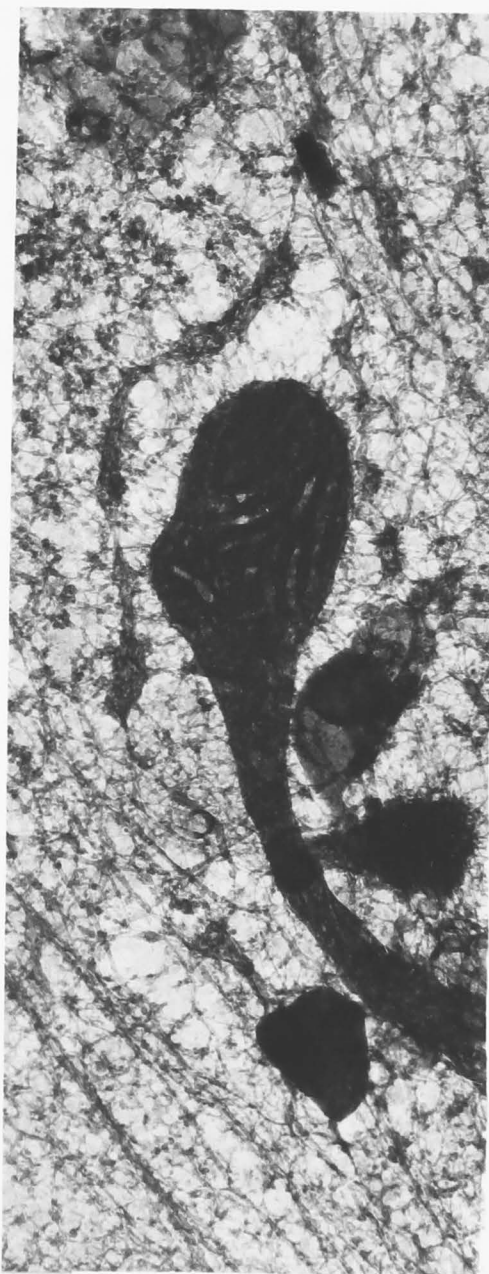


9a

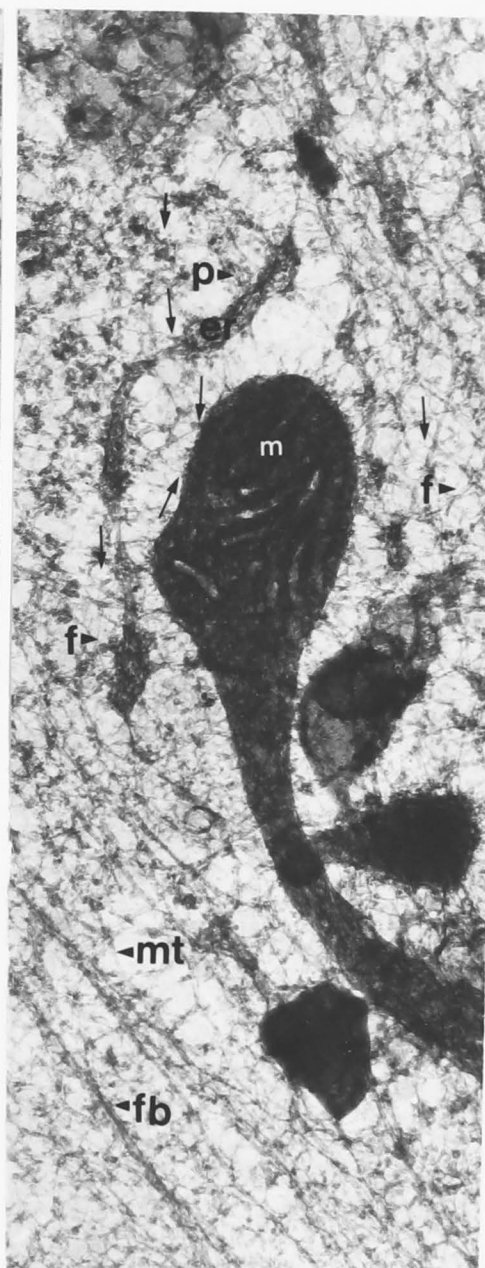


9b

Figs. 10a and b: Stereo pair of electron micrographs showing the arrangement of filaments and organelles in the sub-cortical cytoplasm of a fixed CPD cell. Filament bundles (fb), filaments (f), microtubules (mt), mitochondria (m), endoplasmic reticulum (er) and polyribosomes (p) are readily identifiable. Another feature which is demonstrated here is the contact between the filaments and structures such as mitochondria, endoplasmic reticulum, microtubules and polyribosomes (arrows). The filaments make contact either directly with these structures or through 'node-like' structures. X 54,600.



10a



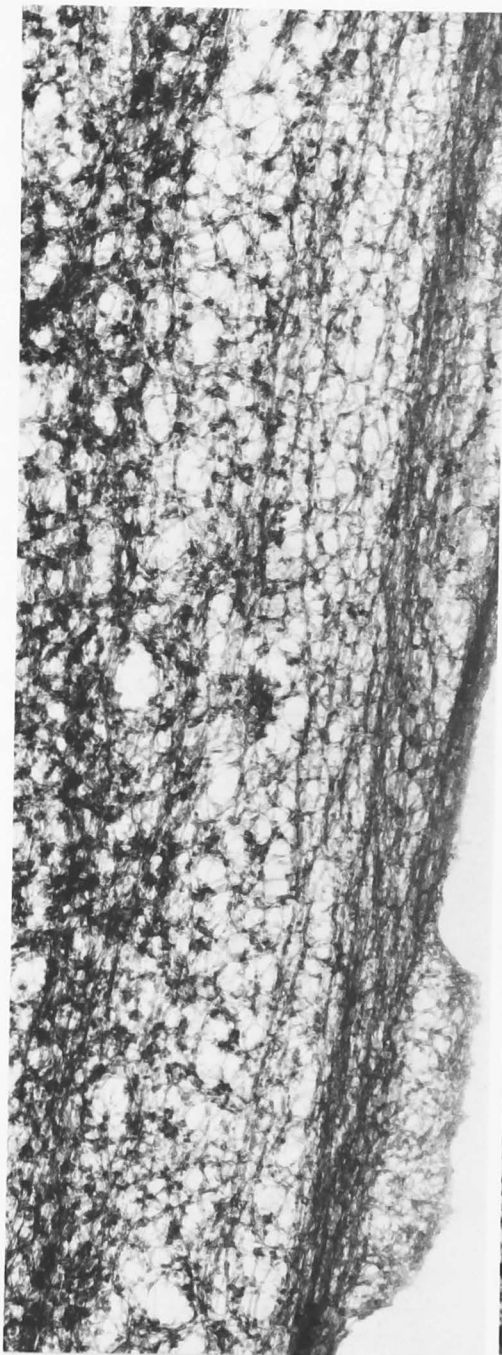
10b



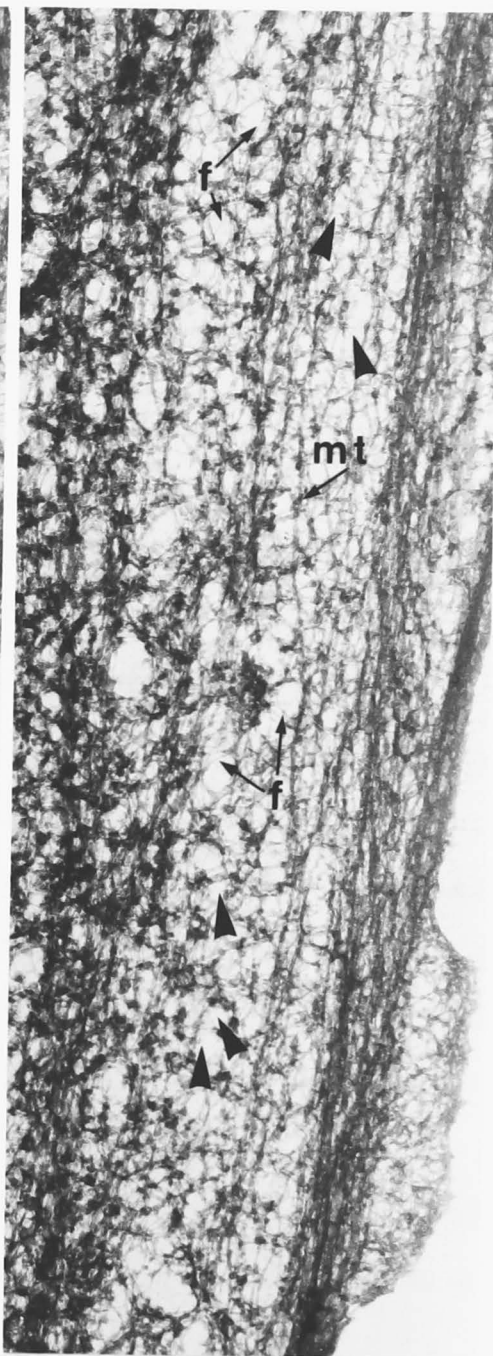
The resultant three dimensional network of filaments surrounds organelles such as mitochondria, polyribosomes and endoplasmic reticulum. The network is also linked to these organelles as well as to microtubules (see below). As shown by stereoscopic viewing, branches arising from intermediate filaments represent true branching and not simply superimpositional images of overlapping filaments.

A separate but equally striking feature of the filament system is the presence of 'node-like' structures which are attached to thin as well as to intermediate filaments. These structures were first described by Buckley (1975) and later in more detail by Buckley and Raju (1976). As illustrated in Figs. 11a and b, the 'node-like' structures are round or ovoid, conical or vase-shaped and exhibit fine tails projecting away from the 'node-like' bodies. The dimensions of the nodes vary depending on their shape. When they are round they measure about 15-16 nm in diameter and the length of the tail ranges from 30-50 nm. Although it is not possible to measure the width of the tails with great accuracy, it is estimated that this is in the range of 1-2 nm. 'Dumb-bell' shaped structures appear to be formed by the tail-to-tail linkage of two of these 'node-like' structures. The 'node-like' structures are distributed throughout the cytoplasm, but their distribution is not uniform. They are concentrated in high densities within the veil-like extensions of the cell margin (Figs. 7a and b). There, the 'dumb-bell' shaped structures extensively cross-link the 4-7 nm filaments thus giving rise to the overall impression of a very fine-mesh filamentous network. The 'node-like' structures are present at moderately high densities within the filament bundles.

Fig. 11a and b: Stereo pair of micrographs showing a portion of a cell clearly demonstrating the presence of numerous short cross-linking structures (arrowheads). These appear as nodes with fine tails. By means of tail to tail attachment, these frequently form 'dumb-bell' shaped structures which cross-link filaments (f) and filaments with microtubules (mt). The nodes are pleomorphic in shape. When they are round they measure 14-16 nm across. The tails are very fine and measure approximately 1-2 nm in diameter and from 30-50 nm in length. X 35,700.



11a



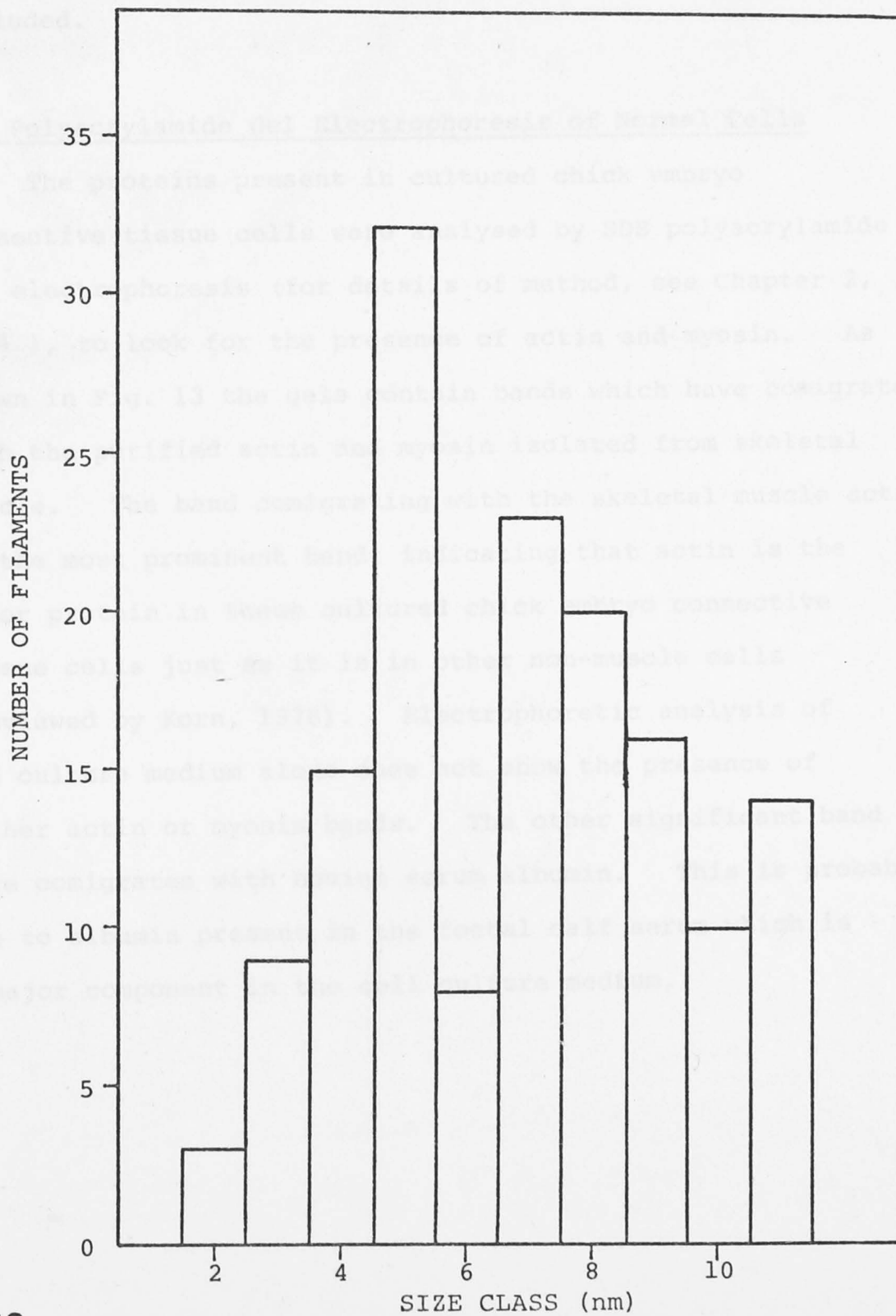
11b

Here they are mostly obscured because of the tight packing of filaments within the bundles. Within the deeper sub-cortical cytoplasm, the 'dumb-bell' shaped structures cross-link the 4-11 nm diameter filaments to one another as well as linking these filaments to other structures such as mitochondria, polyribosomes and microtubules.

(c) *Histogram showing the diameter distribution of the various filaments:* The diameters of the filaments not aggregated into bundles were selected for measurement randomly by dropping a template containing holes onto micrographs of fixed CPD cells and measuring those filaments which lay across the holes. In reality, the filament diameters showed a continuous variation in the range of 2-11 nm. However, for convenience, these measurements are shown in the form of a histogram (Fig. 12). The filaments in the range of 2-3 nm may represent the fine tails of the short interconnecting 'dumb-bell' shaped structures described above. Within the 4-11 nm group of filaments the thin filaments (i.e. those 4-7 nm in diameter) account for 45% of the total and the intermediate filaments (i.e. 7-11 nm in diameter) account for ~ 55% of the total. The relatively high proportion of thin filaments (cf. Ishikawa, Bischoff and Holtzer, 1968) is probably due to the fact that as whole cells were used for the present study, measurements of filaments in the sub-plasmalemmal and cortical regions which are known to have a predominant population of 4-7 nm thin filaments have been included. This technique is in



Fig. 12: Histogram showing the frequency distribution of filament diameters in normal CPD cells. For plotting the histogram those filaments which did not occur in bundles and whose diameters could be determined accurately were measured. As illustrated, there is a continuous range in the diameter distribution of filaments. The 2-4 nm filaments probably represent the short cross-linking elements present throughout the cytoplasm. Based on the classification of others (Ishikawa, Bischoff and Holtzer, 1968; Goldman and Follet, 1969), it is assumed that the 4-11 nm filaments encompass two classes of filaments, namely the 4-7 nm diameter 'thin' filaments and the 7-11 nm 'intermediate'-sized filaments.



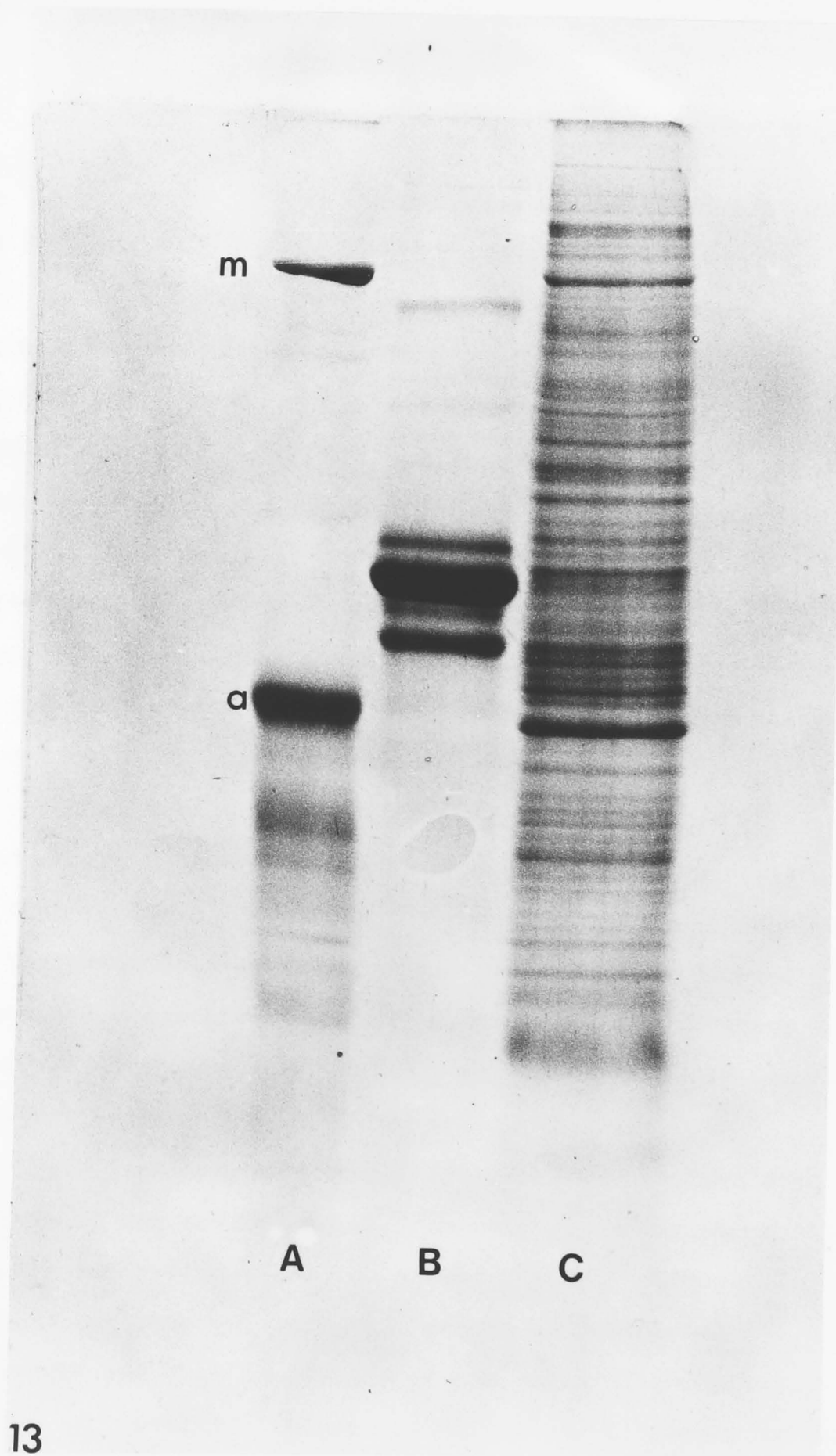
contrast to that of Ishikawa, Bischoff and Holtzer (1968) in which sectioned cells were used and hence most of the cortical and sub-plasmalemmal filaments would have been excluded.

#### SDS Polyacrylamide Gel Electrophoresis of Normal Cells

The proteins present in cultured chick embryo connective tissue cells were analysed by SDS polyacrylamide gel electrophoresis (for details of method, see Chapter 2, p.54 ), to look for the presence of actin and myosin. As shown in Fig. 13 the gels contain bands which have comigrated with the purified actin and myosin isolated from skeletal muscle. The band comigrating with the skeletal muscle actin is the most prominent band, indicating that actin is the major protein in these cultured chick embryo connective tissue cells just as it is in other non-muscle cells (reviewed by Korn, 1978). Electrophoretic analysis of the culture medium alone does not show the presence of either actin or myosin bands. The other significant band here comigrates with bovine serum albumin. This is probably due to albumin present in the foetal calf serum which is a major component in the cell culture medium.

Fig. 13: SDS polyacrylamide gradient (6½-15%) gel electrophoresis (for methods, see p.54 in previous chapter) profile of normal cultured chick embryo connective tissue cells. The cells (C) show the presence of bands which comigrate with the standards (A) consisting of skeletal muscle actin (a) and myosin (m). The culture medium (B) itself does not contain any actin or myosin.





## DISCUSSION

The results of the present chapter can be summarised as follows:

- (1) Light microscopy of living cultured chick embryo connective tissue cells reveals details regarding overall cell shape, structures present within the cell and the form of the cell margins. As determined by direct observation and examination of time-lapse movie records, there is much movement within the cell. Particulate organelles such as mitochondria and lysosomes make rapid movements through the sub-cortical cytoplasm. The veils of the cell margin undergo complex undulatory movements and, from the veils, microvilli extend and retract.
- (2) Lower power electron micrographs of fixed CPD cells demonstrate that the general morphology of living cells, as seen by light microscopy, is not altered significantly by the procedures of fixation and critical point drying. Hence the organelles and other structures seen in phase contrast micrographs of living cells can be readily identified in electron micrographs of CPD cells.
- (3) With regard to the cells' filament systems (the main concern of the present work), the only parts visible with the light microscope are the stress fibres. However, by electron microscopy, the precise form of individual filaments and full complexity of the cells' filament system is revealed. Based on their size, filaments appear

to fall into two categories:

- (i) The thin filaments which measure 4-7 nm in diameter. These are tightly packed into bundles (the stress fibres) or more loosely arranged in sheets. Both are always located within the cortical region of the cytoplasm. Thin filaments also occur as a fine meshwork in the sub-plasmalemmal cytoplasm of the veils. A few thin filaments are found also in the sub-cortical cytoplasm.
  - (ii) The intermediate filaments which measure 7-11 nm in diameter. These occur exclusively in the sub-cortical cytoplasm where they make up 90% of the filaments. Through branching and interconnections with the few sub-cortical thin filaments they form a three dimensional filament network.
- (4) 'Node-like' structures with fine tails are attached to intermediate as well as to thin filaments. Two of these nodes are often found attached, tail to tail, with each other thus forming 'dumb-bell' shaped structures. These cross-link the filaments to one another as well as to other structures such as microtubules, polyribosomes, mitochondria and endoplasmic reticulum.
- (5) SDS polyacrylamide gel electrophoretic analysis of cultured chick embryo connective tissue cells reveals the presence of actin and myosin in the cells.

The main aim of this chapter has been to describe the

three dimensional fine structure of normal, fixed CPD cultured chick embryo connective tissue cells. This has been done with the particular aim of determining details of the form and arrangements of cytoplasmic filaments associated with the moving parts of the cell. Before starting to evaluate the significance of these results, it is extremely important to establish the fidelity of the fixation and CPD procedures used to preserve the native structures of the cell for electron microscopy.

As mentioned earlier, a comparison of low power electron micrographs of fixed, CPD cells with phase contrast micrographs of living cells reveals a close resemblance between the two. The resemblance is strong in areas such as general cell shape, structure and disposition of organelles and stress fibres. Since the resolution of the light microscope is limited, no correlation can be made between high power electron micrographs and the phase contrast images of living cells. However, a comparison between the fine structural images of fixed CPD cells with the published images of cells processed for electron microscopy by conventional embedding and sectioning techniques reveals an extremely close correspondence. With respect to the cell's filament system, the following correlations can be made between the CPD and sectioned cells.

(i) There is a close correspondence between the filament diameters in both CPD and sectioned cells.

(ii) The filament bundles observed in the cortices of fixed CPD cells bear an extremely close resemblance to those seen in sectioned cells (Buckley and Porter, 1967;



Goldman, 1971).

- (iii) The network of filaments found in the veils of CPD cells is similar to that observed in sectioned cells (Yamada, Spooner and Wessels, 1971; Spooner *et al.*, 1973; Wessels, Spooner and Luduena, 1973; Goldman *et al.*, 1976).
- (iv) The 'node-like' structures which are shown to be attached to filaments at irregular intervals in CPD cells were also reported in sectioned cells (Buckley and Porter, 1967).

Thus, it can be reasonably claimed that the procedures of fixation and CPD used in the present work have not produced any artefactual results which are not represented in the sectioned images. The significant advantage of CPD over sectioning for the present purpose is the possibility of readily acquiring a clear understanding of the three dimensional architecture of cultured cells.

A detailed knowledge of the system of filaments, their distribution and the structures closely related to them is useful only if the relationship between the various filaments and the movements exhibited by different parts of the cell can be established. Using the results described in this chapter and the previously published observations of subcellular movements, this can be achieved. The sub-plasmalemmal cytoplasm, which occupies the rapidly moving veil-like extensions of the cell margin, contains a fine meshwork of filaments cross-linked by node-like structures with fine tails. No other structures are seen

in this cytoplasm. In cultured macrophages, Axline and Reaven (1974) demonstrated the relationship between parallel sub-plasmalemmal filaments and movements of plasma membrane such as phagocytosis. Hence, it seems highly likely that these sub-plasmalemmal filaments are directly concerned with the rapid movements carried out by the veils.

With respect to stress fibres (filament bundles), these may form and disappear according to the needs of cell motility. For example, it is believed that stress fibre formation is an essential feature of certain gradual processes such as the spreading of cultured cells (Goldman *et al.* 1976). However, with movie records taken at 2 frames per second, Buckley and Porter (1967) showed that, compared with all other sub-cellular movements, the changes in stress fibre dimensions and positions are extremely slow. Nevertheless, the same authors also showed, with time-lapse movie records taken over periods of many hours, that the intracellular dispositions of stress fibres undergo major changes.

In the sub-cortical cytoplasm, particulate organelles such as the mitochondria and lysosomes, which show active movements, are surrounded by a three dimensional network of filaments. In some instances it appears that these organelles are linked to the filaments through the 'node-like' structures with fine tails. Buckley (1974) showed that hypertonic solutions can arrest the movements of organelles within the cultured cells. Electron microscopic images of those cells revealed reduced numbers of sub-cortical

filaments which normally surround the organelles. Thus, there appears to be a correlation between the presence of filaments in the sub-cortical region and various movements of organelles which occur in this region.

It is widely assumed that the various non-muscle cell movements are brought about by actin-myosin based contractile interactions (Pollard and Weihing, 1974). This idea is supported by the presence of a large quantity of actin and a relatively small quantity of myosin in cultured chick embryo connective tissue cells (as shown by SDS polyacrylamide gel electrophoresis). However, if the cytoplasmic filaments are responsible for subcellular movements, the first and foremost task should be to localize these contractile proteins within the cell's filament system. As mentioned in Chapter 1, attempts have been made by others to localize contractile proteins within various non-muscle cells (reviewed by Pollard and Weihing, 1974).

Immunofluorescence techniques have been applied to localize actin (Lazarides and Weber, 1974; Goldmen *et al.*, 1975), myosin (Weber and Groeschel-Stewart, 1974) and  $\alpha$ -actinin and tropomyosin (Lazarides, 1976). However, the precision of these localizations is limited to the resolving power of the light microscope. Identification of actin at the ultrastructural level was made possible by the HMM-labelling technique first developed by Huxley (1963). Ishikawa, Bischoff and Holtzer (1969) applied this technique to localize actin in non-muscle cells.

However, a complete picture of the distribution of filamentous actin in non-muscle cells has yet to be attained

and, to date, there have been no precise techniques developed for the ultrastructural identification of the other 'muscle' proteins. The future chapters of this thesis will be devoted to the examination of this problem.

#### CHAPTER 4 - IDENTIFICATION AND LOCALIZATION OF $\beta$ -ACTIN IN CYTOSOLIC FILAMENTS: 1. STUDIES USING HEAVY MERCURY



## INTRODUCTION

Heavy meromyosin (HMM) is a proteolytic fragment of the myosin molecule obtained after tryptic digestion (Szent-Gyorgyi, 1953). This fragment consists of the head and a portion of the tail of the myosin molecule (Fig. 1). It was first demonstrated by Huxley (1953), when HMM was added to a preparation of F-actin, the head of the myosin molecule was seen to project from the filaments in a regular manner resulting in the configuration resembling that of an  $\alpha$ -helix series of

## CHAPTER 4 - IDENTIFICATION AND LOCALIZATION OF F-ACTIN IN CYTOPLASMIC FILAMENTS:

### I. STUDIES USING HEAVY MEROMYOSIN



Fig. 1. Diagrammatic representation of myosin molecule showing the site of action (arrow) of trypsin which cleaves myosin into HMM and LMM fragments.

## INTRODUCTION

Heavy meromyosin (HMM) is a proteolytic fragment of the myosin molecule obtained after tryptic digestion (Szent-Gyorgyi, 1953). This fragment consists of the head and a portion of the tail of the myosin molecule (Fig. 1). As first demonstrated by Huxley (1963), when HMM is added to a preparation of F-actin, the head portions bind to the actin filaments in a regular manner resulting in the projection of the tails away from the filaments, the overall configuration resembling that of an in-line series of arrowheads (Huxley, 1963; Moore, Huxley and De Rosier, 1970). To date, this technique provides the most specific marker for identifying actin filaments within cells.

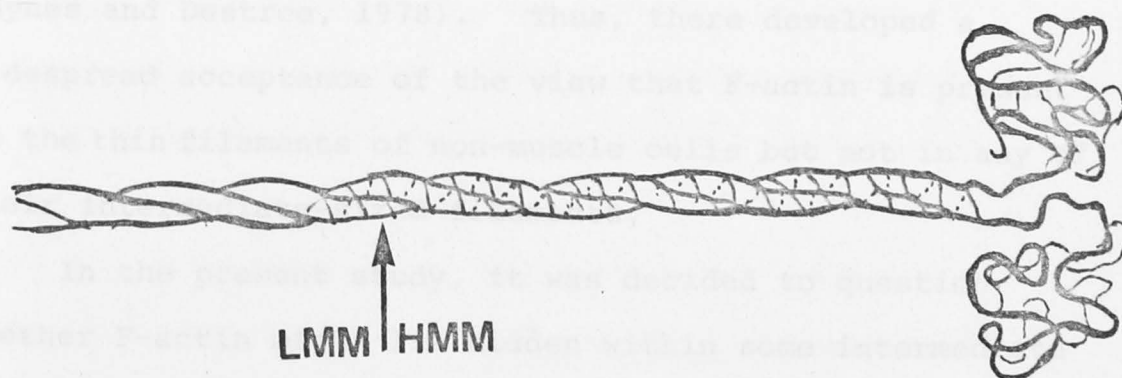


Fig. 1: Diagrammatic representation of myosin molecule showing the site of action (arrow) of trypsin which cleaves myosin into HMM and LMM fragments.

HMM labelling was first used to localize actin filaments in non-muscle cells by Ishikawa, Bischoff and Holtzer (1969). These authors found that in non-muscle cells, such as cultured fibroblasts, thin filaments (5-7 nm in diameter) which predominated in the cortical regions of the cells labelled with HMM. Based on their diameter (identical to the thin (actin) filaments of muscle) and their ability to label with HMM, these were considered to be actin-containing filaments. However, most of the sub-cortical filaments, measuring 8-12 nm in diameter, (Ishikawa, Bischoff and Holtzer, 1968) were intermediate in size between the thin actin filaments and the thick myosin filaments of muscle and these intermediate filaments did not label with HMM. Accordingly these were considered not to contain actin. This finding was later confirmed by many other workers (e.g. Goldman and Knipe, 1973). Additional evidence which seemed to support this conclusion was also obtained from antibody-fluorescence studies (Hynes and Destree, 1978). Thus, there developed a widespread acceptance of the view that F-actin is present in the thin filaments of non-muscle cells but not in any of their intermediate-sized filaments.

In the present study, it was decided to question whether F-actin might lie hidden within some intermediate filaments of cultured connective tissue cells for the following reasons. As seen in the sub-cortical cytoplasm of critical-point dried (Buckley and Raju, 1976; see also previous chapter), sectioned and negatively stained cells (Buckley, 1975) intermediate and thin filaments are connected

in the form of a three dimensional network. In addition, tiny 'node-like' structures which may represent cytoplasmic myosin (see chapter 7 and also Buckley and Raju, 1976) are, in the non-myosin extracted cultured chick embryo fibroblast, attached to intermediate as well as to thin filaments.

To answer the question of whether or not some intermediate filaments contain actin it was decided to reinvestigate the binding of HMM to filaments within glycerinated myosin-extracted cultured chick embryo connective tissue-cells, by varying the conditions of treatment prior to the application of HMM. It was thought possible that the presence of some protein(s) on the surface of intermediate filaments could (if F-actin were present within) block the binding of HMM. With this possibility in mind, glycerinated, myosin-extracted cells were given a mild pre-treatment with trypsin before the HMM labelling step. This was done in the hope that the trypsin would remove possible blocking proteins from the intermediate filaments, thereby exposing any underlying F-actin (known to be relatively trypsin insensitive (Tilney, 1977)) to the HMM. Purified muscle actin filaments and  $Mg^{2+}$  paracrystals of actin were also labelled with HMM to obtain an appreciation of the binding pattern of HMM to both single actin filaments and to actin filaments in bundles. It was hoped to utilize this knowledge in interpreting the results obtained with HMM labelling of filaments occurring within the cell.

The experimental results to be described in this chapter



will be presented in the following order:

HMM Labelling of Purified Muscle Actin Filaments

- (a) F-actin filaments;
- (b)  $Mg^{2+}$  paracrystals of F-actin.

HMM Labelling of Glycerinated, Myosin-extracted Cells

- (a) Ultrastructure of cells without HMM;
- (b) Ultrastructure of cells with HMM.

HMM Labelling of Trypsin-treated, Glycerinated, Myosin-extracted Cells

- (a) Ultrastructure of cells without HMM;
- (b) Ultrastructure of cells with HMM.

RESULTS

HMM Labelling of Purified Muscle Actin Filaments

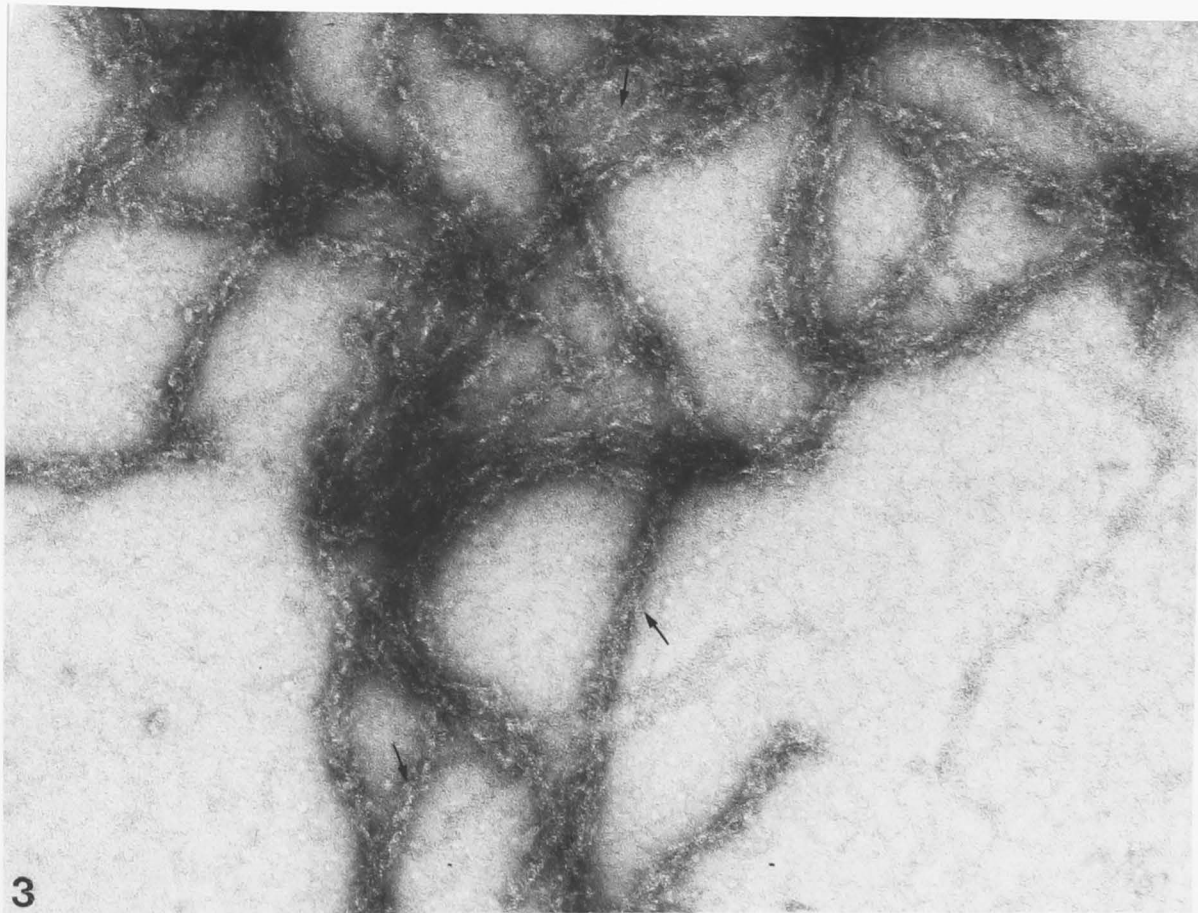
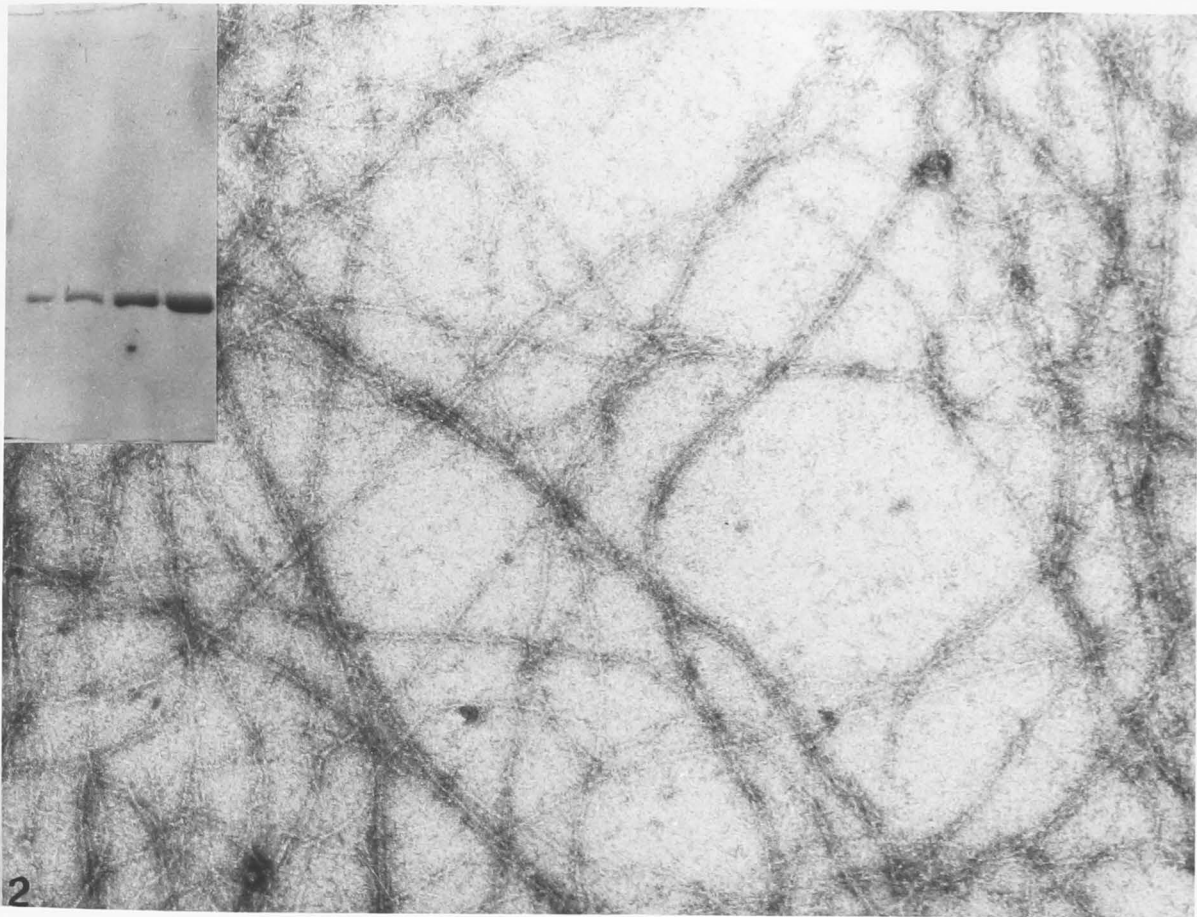
(a) *F-actin filaments:* The appearance of unlabelled muscle F-actin filaments is illustrated in Fig. 2. The filaments are long and occur both singly and, associated together, in a multistranded form. Single filaments measure  $\sim 6$  nm in diameter. When treated with HMM (for details of treatment, see Chapter 2, p. 43) all the filaments became decorated. At very low magnifications such decorated filaments appeared increased in thickness and somewhat fuzzy in outline. With higher magnifications, as shown in Fig. 3, this fuzziness could be resolved as distinct arrowheads. These arrowheads have a regular periodicity of approximately 37 nm.

(b)  *$Mg^{2+}$  paracrystals of F-actin:* As known from the work

Fig. 2: Electron micrograph of a negatively stained preparation of purified skeletal muscle F-actin. The filaments occur either singly or in a multistranded form. Single filaments measure  $\sim 6$  nm in diameter and when filaments are in a multistranded form they measure up to 21 nm. X 72,000.

The inset shows an SDS polyacrylamide gel electrophoretic profile of a sample used in the above ultrastructural study. Samples were run on a 10% gel at a constant current of 34 mA using the methods described in Chapter 2 (p.54).

Fig. 3: Electron micrograph of negatively stained preparation of F-actin treated with HMM (for methods, see Chapter 2, p.43). The filaments no longer appear smooth, but show arrowhead formations. This arrowhead pattern is caused by the more or less constant angle of attachment of HMM to the underlying actin filaments (arrows). The periodicity of arrowheads is  $\sim 37$  nm. X 119,000



of Hanson (1973), an increase in  $Mg^{2+}$  concentration above  $\sim 20$  mM results in side to side alignment of actin filaments, the subunit cross over points of which may then occur in register (Fig. 4 inset). The number of filaments present within each single paracrystal varies greatly. As illustrated in Fig. 4, branching of paracrystals may occur through separations of the F-actin strands within the paracrystal bundles.

When  $Mg^{2+}$  paracrystals are treated with HMM (Fig. 5) it is not possible to resolve individual arrowheads because of the close packing of filaments with resultant overlapping of arrowheads. However, towards each end of the paracrystals, where the filaments diverge away from one another, the arrowhead pattern can be clearly appreciated (Fig. 6).

#### HMM Labelling of Glycerinated, Myosin-extracted Cells

(a) *Ultrastructure of cells without HMM:* The fine structure of glycerinated, myosin-extracted cells is described in detail in Chapter 7 which deals with the localization of myosin. In that chapter, however, the descriptions are applied to cells processed for electron microscopy by critical-point drying. Here, descriptions will be based on the structure of glycerinated, myosin-extracted cells which have been negatively stained. This is because it was established that the negative staining procedure, unlike critical-point drying, provided a sensitive indication of HMM labelling of actin filaments within cells. Figs. 7 and 8 illustrate different regions of glycerinated, myosin-extracted cells which have been negatively stained.



Fig. 4:  $\text{Mg}^{2+}$  paracrystal of F-actin (prepared as described in Chapter 2, p.43) showing the side by side alignment of actin filaments. The paracrystals are generally long and, by splitting, aggregates of actin filaments sometimes diverge away to form branches (arrow). Inset shows the crossing-over points of the actin subfilaments occurring in register (arrowheads) which are associated with the close side by side alignment of actin filaments. This arrangement gives a regular periodicity to paracrystals. X 91,000; inset X 183,000.

Fig. 5: When the  $\text{Mg}^{2+}$  paracrystals of F-actin are treated with HMM, the filaments no longer appear smooth as in the control (Fig. 4). However, although there is a strong suggestion of binding of HMM to filaments within the paracrystal (arrows), individual arrowheads cannot be clearly resolved. X 45,000.

Fig. 6: Towards each end of HMM-treated paracrystals where the filaments are more loosely packed the HMM-binding, with resultant arrowheads, is more distinctly seen. (arrows). X 108,000.

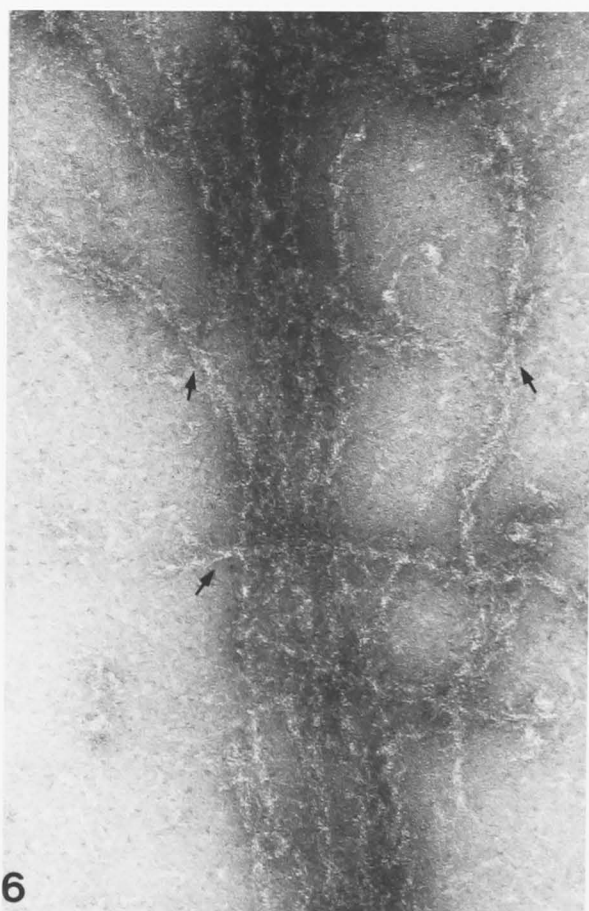
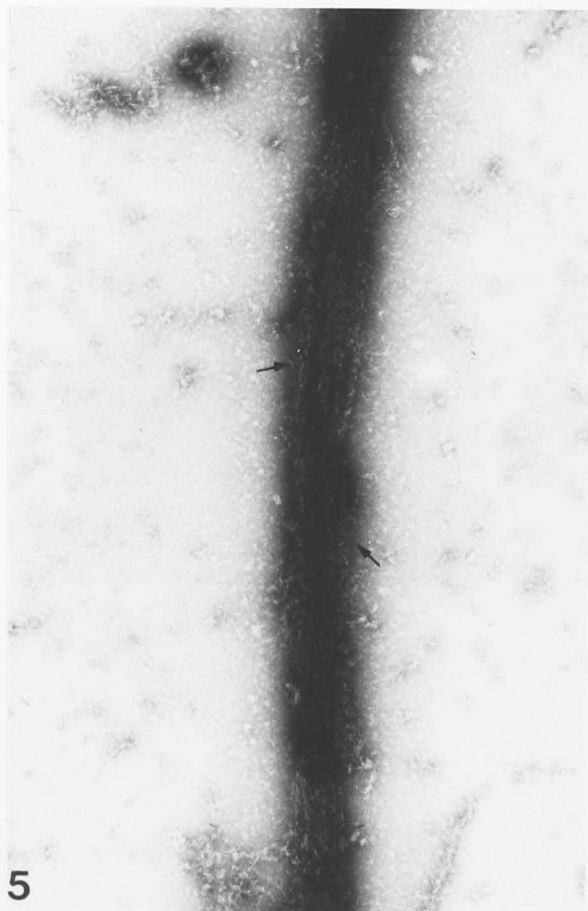
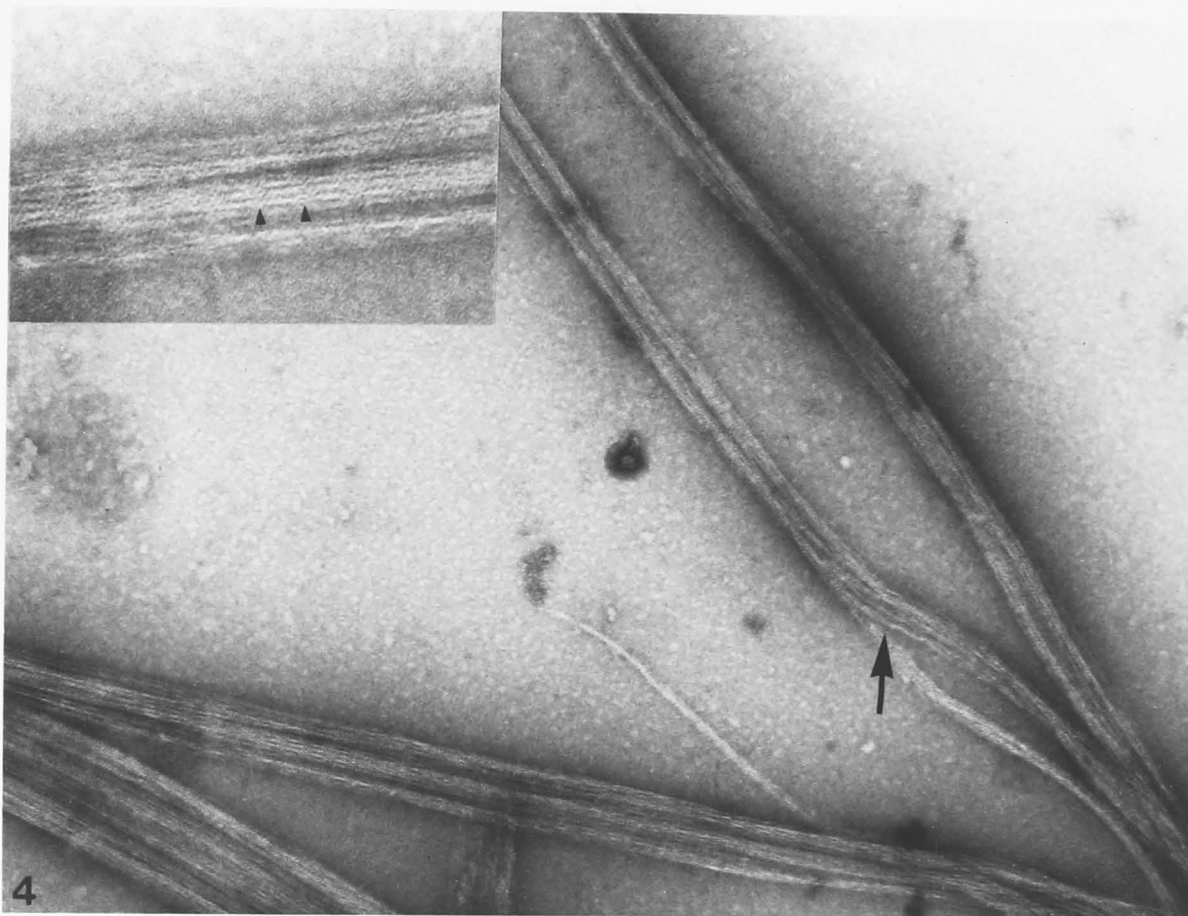
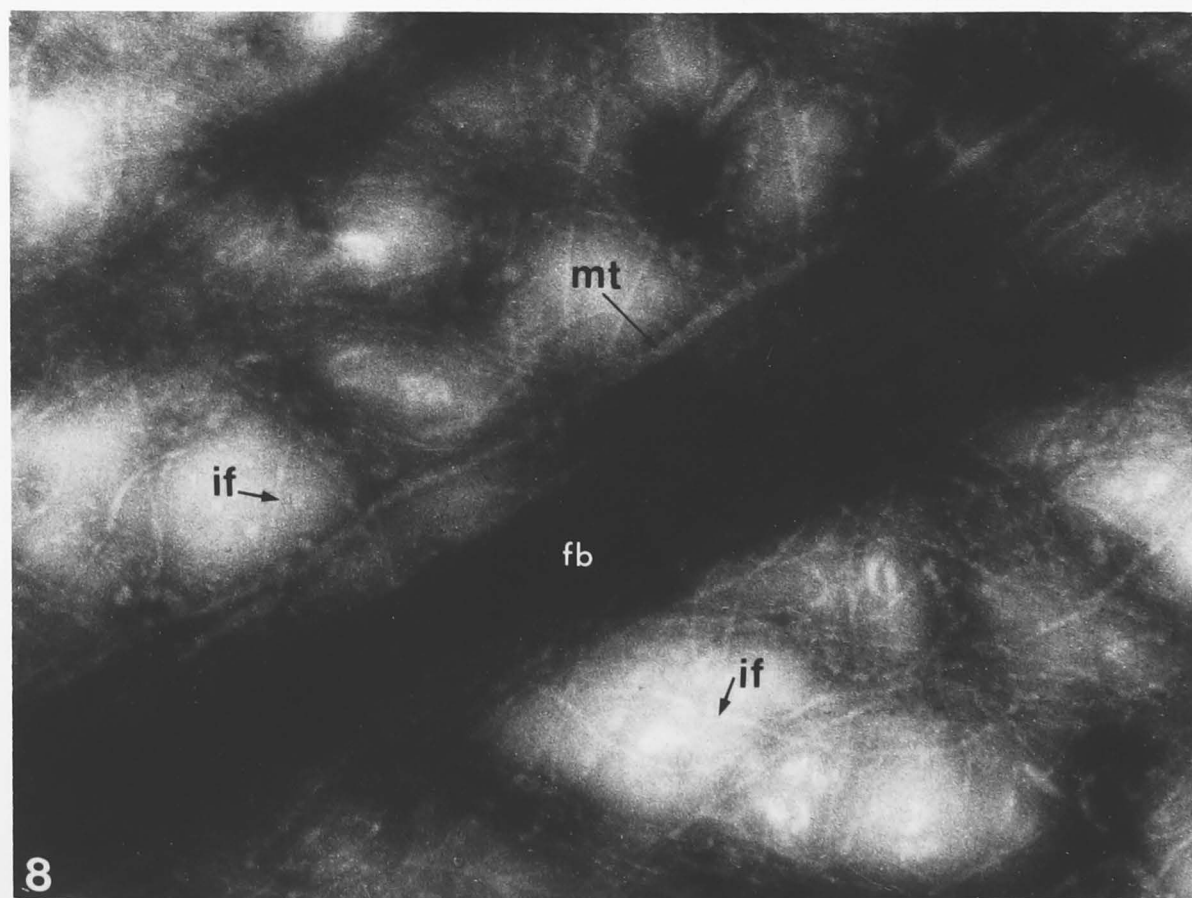
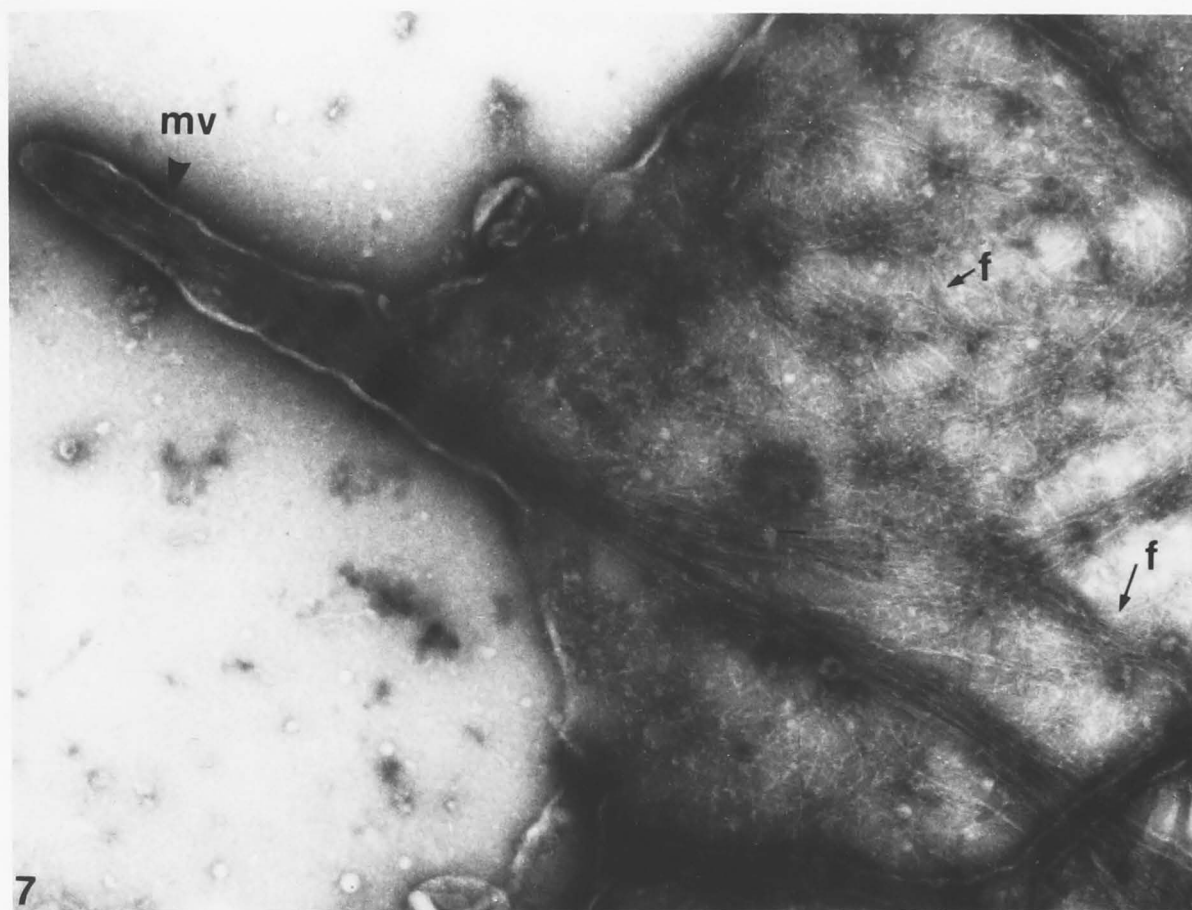


Fig. 7:Electron micrograph of a negatively stained preparation of a glycerinated, myosin extracted cell (for methods, see Chapter 2, p.32) showing the highly motile veil-like extension of the cell margin. A finger-like microvillus (mv) can be seen projecting out from the veil. The filaments (f) in the veil measure 4-7 nm in diameter. Here they are arranged in the form of a loose network. The microvillus contains a dense core of more or less closely aligned 4-7 nm filaments. X 70,000

Fig. 8:Electron micrograph of a portion of a negatively stained, glycerinated, myosin-extracted cell showing the arrangement of filaments in the deeper cytoplasm. Filament bundles (fb) consisting of 4-7 nm diameter thin filaments can be seen. Intermediate filaments (if) measuring 7 -11 nm in diameter are meandering through the cytoplasm between the bundles. This micrograph also shows degenerating microtubules (mt). Microtubules are not commonly seen in glycerinated, myosin-extracted cells because these procedures are carried out at 4°C. X 70,000.





In Fig. 7, the arrangement of filaments within the highly motile veil-like extension of a cell is depicted. Such veils contain thin filaments, 4-7 nm in diameter, which are arranged in the form of a loose meshwork. A loose bundle of these filaments forms the core of microvilli.

Fig. 8 shows a portion of another glycerinated, myosin-extracted cell. Here there are several cortical filament bundles, consisting of parallel arrays of 4-7 nm thin filaments, separated by loosely arranged intermediate (7-11 nm) filaments. These intermediate filaments branch and anastomose with one another to form a network. The true nature of this branching is revealed only by stereoscopic viewing of critical-point dried cells (see Chapter 7). Microtubules are occasionally present, but here they are not long and continuous as in normal cells.

(b) *Ultrastructure of cells with HMM:* The HMM labelling pattern found was similar to that obtained by other workers (Ishikawa, Bischoff and Holtzer, 1969; Allison, Davies and De Petris, 1971; Goldman and Knipe, 1973). As shown in Fig. 9, almost all filaments in the veil labelled with HMM and individual arrowheads can be easily appreciated. However, in the case of filament bundles, even though the filaments within the bundles show definite binding, it is hard to distinguish individual arrowheads because of tight packing of filaments within the bundles (Fig. 10). The striking feature is the absence of labelling of intermediate filaments lying in between the bundles. These intermediate filaments appear smooth, as in the control (non-HMM treated)

Fig. 9: Electron micrograph showing the veil of a glycerinated, myosin-extracted cell which has been treated with HMM (for methods, see Chapter 2, p.33). Cell margins (cm) and filament bundles (fb) form the boundaries of the veil. Almost all the filaments in the veil are labelled with HMM. While most of the filaments appear fuzzy, arrowhead patterns can be clearly seen in some areas (arrows). X 70,000.

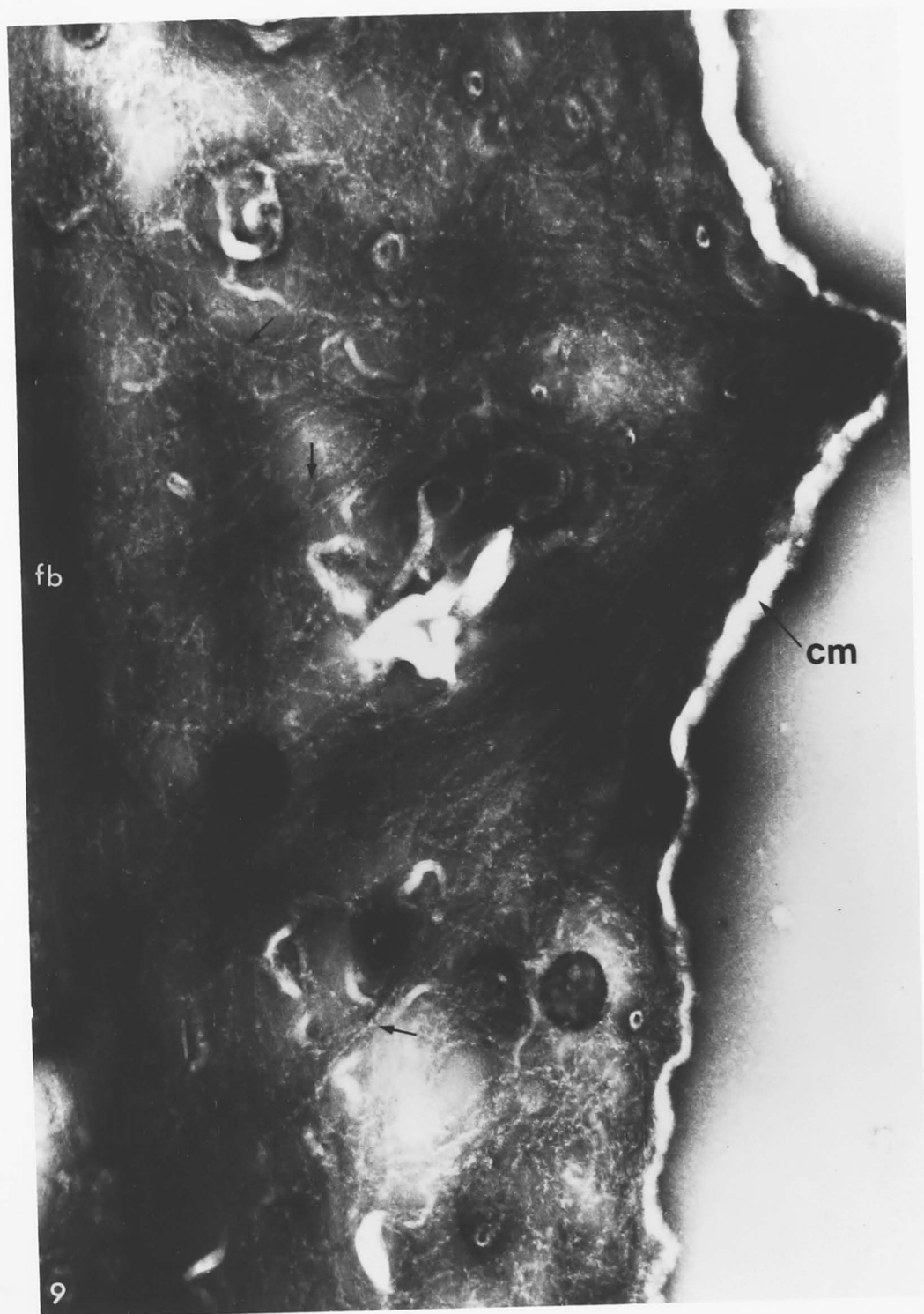
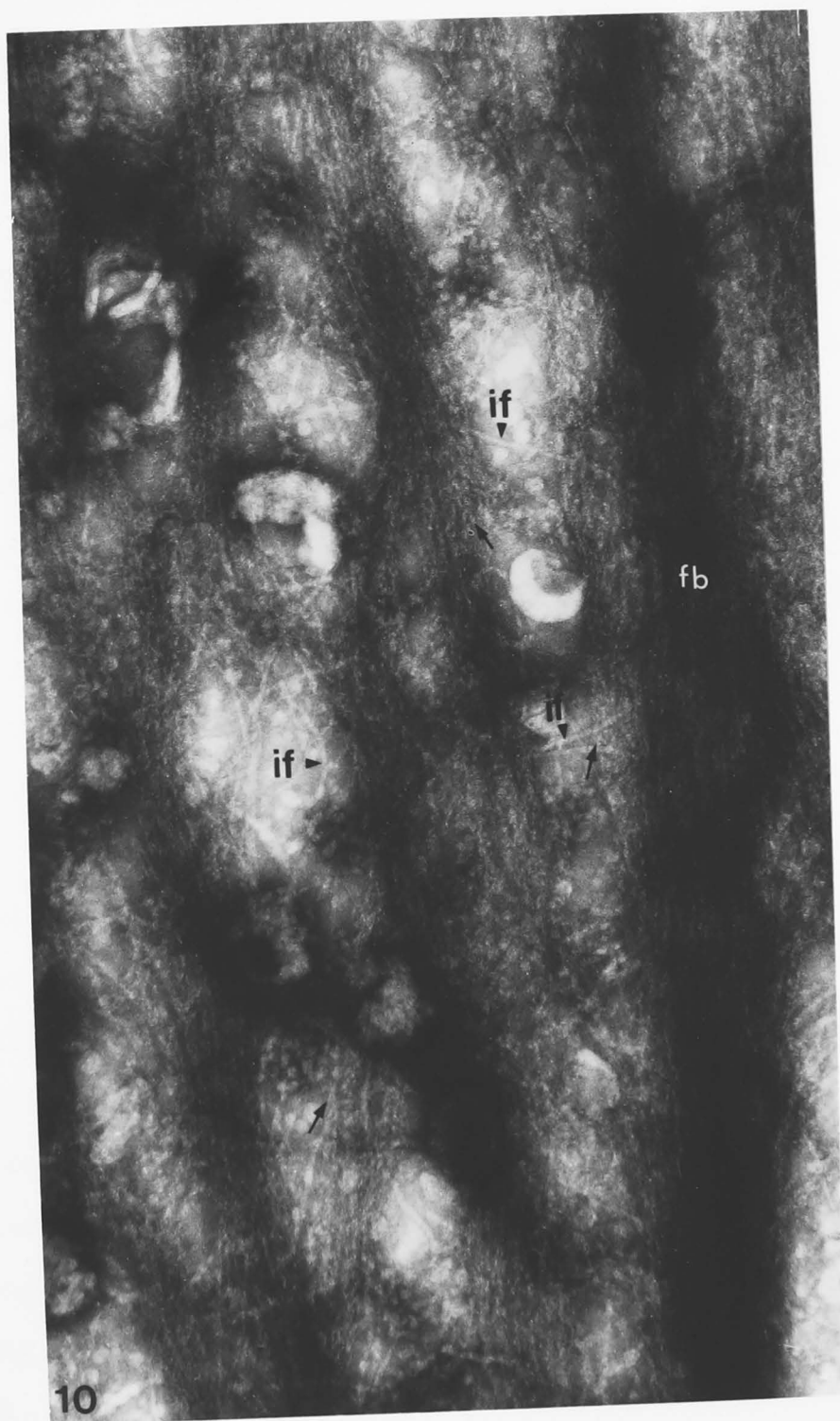


Fig. 10: Electron micrograph showing a portion of a glycerinated, myosin-extracted cell which has been treated with HMM. The filaments present within the bundles (fb) show binding of HMM and hence they appear fuzzy. In a few areas, the arrowhead pattern can be clearly seen (arrows). The most interesting feature is the non-binding of HMM by intermediate filaments (if). These filaments appear smooth as in the control cells (cf. Fig. 8). X 70,000.





cells.

HMM Labelling of Trypsin-treated, Glycerinated,  
Myosin-extracted Cells

(a) *Ultrastructure of cells without HMM:* As illustrated in Fig. 11, the mild trypsin treatment does not result in the loss of intermediate filaments. The characteristic meandering intermediate filaments running in between the bundles, appearing as in non-trypsin treated cells, are still obvious in these trypsin treated cells. The continued presence of thin filament bundles and other features of intermediate filaments (branching and a wide range of diameters) are as seen in non-trypsin treated cells.

Because of reports that intermediate filaments disappear altogether from some types of cell when treated with proteolytic enzymes (Small and Sobieszek, 1977), it was decided to investigate in more detail possible effects of trypsinization on the diameters and numbers of intermediate filaments in the connective tissue cells being studied. To do this, the diameter distributions of sub-cortical filaments in a group of glycerol myosin-extracted cells were compared with those in a group which had received the additional trypsin treatment. Filaments from each group were measured without subjective bias by withholding the identity of micrographs from observers (Buckley, Raju and Stewart, 1978). Filaments for measurement were selected randomly by dropping a template with holes onto each print.

The resulting diameter distributions of the filaments are presented in the histograms (Fig. 12). Statistical

Fig. 11: Portion of a negatively stained cell which was gently trypsinized after glycerination and myosin extraction (for methods, see Chapter 2, p.34). The cell margin (cm) and filament bundles (fb) are easily identifiable. In addition to these features, the meandering 7-11 nm diameter intermediate filaments (if) are clearly distinguishable. They show a close resemblance to the intermediate filaments seen in non-trypsinized cells (cf. Fig. 8). X 70,000.

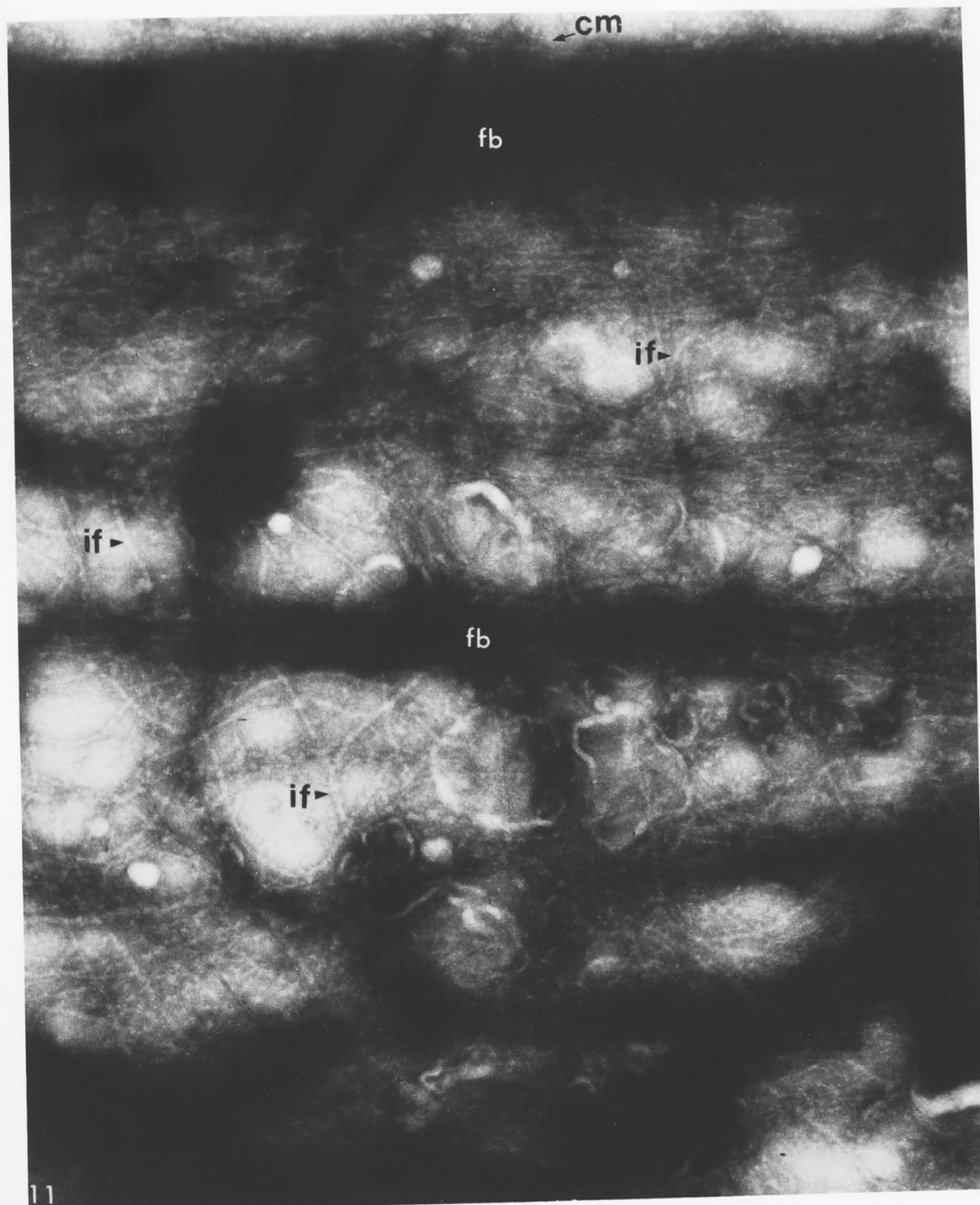
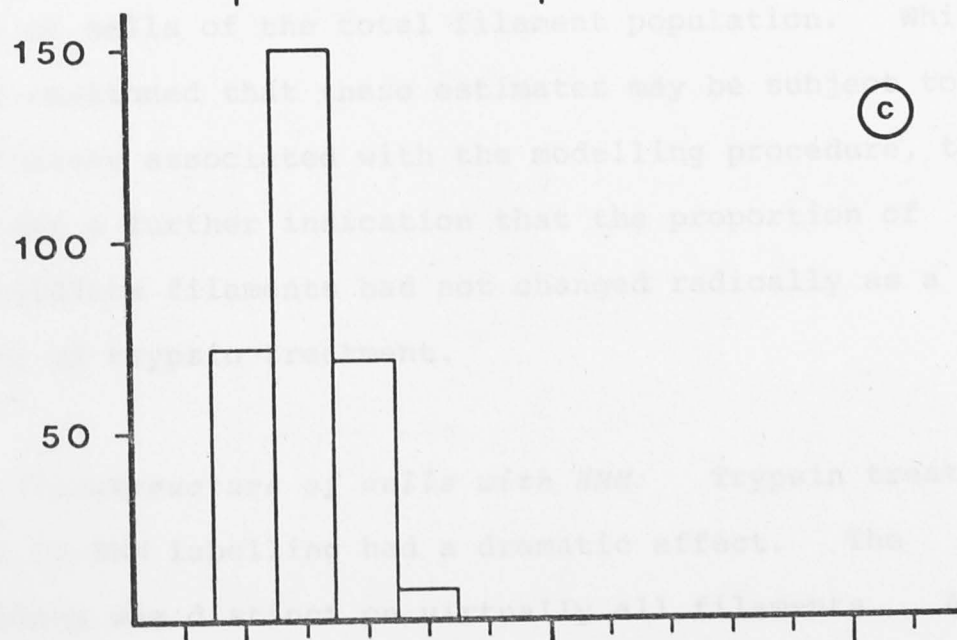
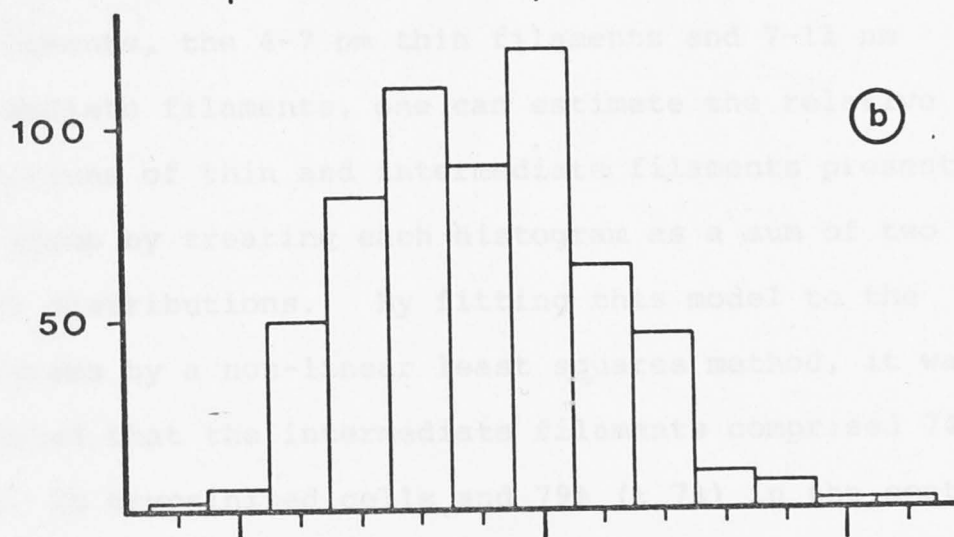
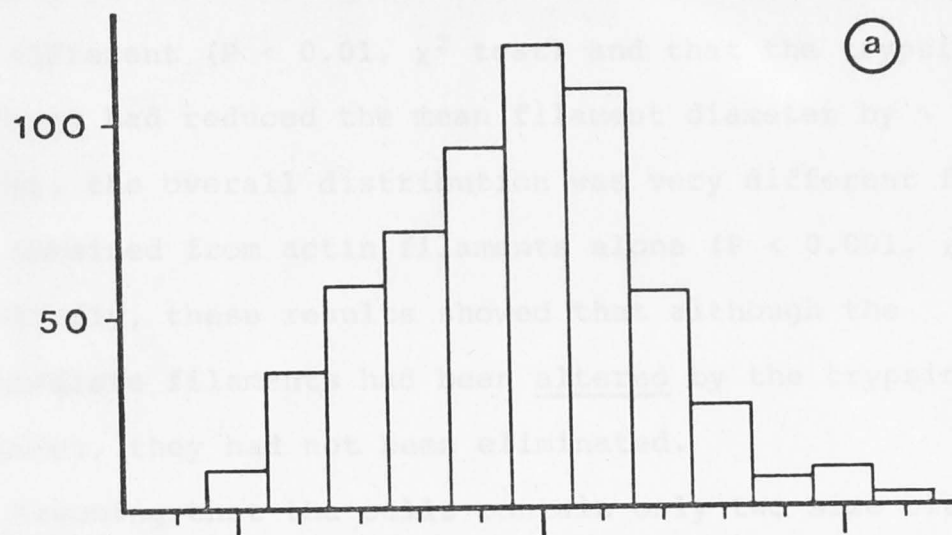




Fig. 12: Histograms of filament diameters.

- (a) Cytoplasmic filaments without trypsin treatment.
- (b) Cytoplasmic filaments after trypsin treatment.
- (c) Rabbit skeletal muscle F-actin.

Frequency



12

5

10

15

Diameter (nm)

analysis of this data indicated that these histograms were different ( $P < 0.01$ ,  $\chi^2$  test) and that the trypsin treatment had reduced the mean filament diameter by  $\sim 7\%$ . However, the overall distribution was very different from that obtained from actin filaments alone ( $P < 0.001$ ,  $\chi^2$  test). Accordingly, these results showed that although the intermediate filaments had been altered by the trypsin treatment, they had not been eliminated.

Assuming that the cells contain only two size classes of filaments, the 4-7 nm thin filaments and 7-11 nm intermediate filaments, one can estimate the relative proportions of thin and intermediate filaments present in each group by treating each histogram as a sum of two normal distributions. By fitting this model to the histograms by a non-linear least squares method, it was estimated that the intermediate filaments comprised 74% ( $\pm 7\%$ ) in trypsinized cells and 79% ( $\pm 7\%$ ) in the control group of cells of the total filament population. While it is cautioned that these estimates may be subject to uncertainty associated with the modelling procedure, they do offer a further indication that the proportion of intermediate filaments had not changed radically as a result of trypsin treatment.

(b) *Ultrastructure of cells with HMM:* Trypsin treatment prior to HMM labelling had a dramatic effect. The labelling was distinct on virtually all filaments. As Fig. 13 indicates, as before, the parallel thin filaments lying in the bundles are labelled with HMM. However, the

Fig. 13: Electron micrograph of a portion of a cell which was treated with HMM after trypsinization. All filaments are labelled with HMM: not only the filaments in bundles but also the long intermediate filaments meandering between the bundles show clear arrowhead patterns (cf. Fig. 10). X 54,000.





most striking feature is the labelling of the diversely oriented filaments of the sub-cortical cytoplasm which show clear arrowhead patterns. The distinctness of this binding is illustrated in a higher magnification micrograph (Fig. 14). It is not uncommon to see a labelled intermediate filament dividing to form two branches, both of which are also clearly labelled with HMM (Buckley, Raju and Stewart, 1978).

To obtain a more quantitative appreciation of the increase in the proportion of filaments exhibiting HMM labelling after the trypsin treatment, a protocol similar to that employed to investigate changes in filament diameter was used. Using a sampling template, 20 filaments were selected from each of 10 micrographs taken from trypsin-treated and non-trypsin-treated groups of cells. Filaments were categorized according to whether or not they bound HMM. In the control (untrypsinized) cells, 113 filaments were HMM-labelled and 87 were unlabelled. By contrast, in the trypsin-treated cells, 195 filaments labelled and only 5 did not. This difference is significant at the 0.01% level ( $\chi^2$  test).

#### DISCUSSION

The major conclusion of the above-described experiments is that in cultured chick embryo connective tissue cells not only do all the thin filaments (present in the sub-plasmalemmal cytoplasm and cortical bundles) contain F-actin but so also do most of the intermediate filaments in

Fig. 14: A higher magnification electron micrograph of a cell, which was subjected to the same treatments as that shown in Fig. 13, showing the labelling of all filaments. The characteristic arrowhead pattern is clearly recognizable. X 76,000.





the sub-cortical cytoplasm. The reasons for arriving at this conclusion are as follows:

- (1) The 7-11 nm diameter sub-cortical intermediate filaments, which show very little or no labelling in non-trypsin-treated cells, show characteristic labelling in trypsin-treated cells.
- (2) Control preparations showed that the trypsin treatment itself had no significant effect in eradicating these filaments, since this treatment allowed the survival of the great majority of intermediate filaments.

The labelling of 4-7 nm diameter thin filaments is an expected result, as many workers (e.g. Ishikawa, Bischoff and Holtzer, 1969; Allison, Davies and De Petris, 1971; Goldman, 1975) have reported this previously. Hence, the conclusion that F-actin is present in the thin filaments is simply consistent with the findings of others. However, the labelling of intermediate-sized filaments with HMM is a totally new and unexpected result. Because of the known highly specific affinity between HMM and actin (Huxley, 1963), the only explanation of the present results which seems possible is that in cultured chick embryo connective tissue cells, most of the intermediate filaments contain F-actin. This observation that intermediate filaments label with HMM differs from that of Ishikawa, Bischoff and Holtzer (1969) and Goldman and Knipe (1973). However, it should be stressed that the positive result of HMM labelling of intermediate filaments reported here occurred only after trypsin pre-treatment, a situation not previously explored by other investigators.

A possible explanation of this result is that trypsin degrades intermediate filament accessory protein(s) which normally block the binding of HMM to underlying actin filaments. This interpretation has led to the proposal of a model for intermediate filament structure based on the idea of a multistranded ( $\sim 2-5$ ) F-actin core associated with accessory proteins (Buckley, Raju and Stewart, 1978). The finding that purified muscle F-actin by itself can form multistranded arrays which branch and anastomose with one another (see Fig. 2 in this chapter) supports the feasibility of the model as far as the polymerized structure of actin is concerned. Some sort of multistrandedness within the cell's sub-cortical filament system is suggested by the variable widths of the intermediate filaments (Ishikawa, Bischoff and Holtzer, 1968) and by the anastomosing of intermediate filaments, including the branching of thin filaments from intermediate filaments (Buckley and Raju, 1976).

Blocking of actin filaments from labelling with HMM by accessory proteins is not a new finding. Tilney (1975) found that in the acrosomal process of Limulus sperm, a specific protein associated with F-actin can have such an HMM-blocking effect. These results are extremely important because the molecular weight of the HMM blocking protein in Limulus (55,000) is very similar to the major protein found to be characteristic of intermediate filaments in many types of vertebrate non-muscle cells (Gordon, Bushnell and Burrridge, 1978; Starger *et al.*, 1978). The association between actin and intermediate filament-specific

accessory protein(s) is also suggested by the work of others showing that whenever attempts were made to isolate intermediate filament proteins, actin copurified (Lazarides and Hubbard, 1976; Starger and Goldman, 1977; Lehto, Vertanen and Kurki, 1978; Franke *et al.*, 1978). It should be stressed, however, that actin might not have any role to play at all in the composition of the intermediate filaments of cells such as neurones (Shelanski, Yen and Lee, 1976). In some cells (e.g. chicken gizzard smooth muscle) actin may be present in the intermediate filaments, but not necessarily in a filamentous form (see Chapter 7). Accordingly, it will be necessary to investigate the intermediate filaments of each type of cell to determine which contain actin and which do not and, of those that contain actin, whether or not it occurs in the F-actin form.

Most of the recent work on structural analyses of intermediate filaments is based on immunofluorescence studies involving antibodies (Blose, Shelanski and Chacko, 1977; Osborn, Franke and Weber, 1977; Hynes and Destree, 1978; Gordon, Bushnell and Burridge, 1978). The results of these studies convincingly demonstrate that the characteristic fluorescence of intermediate filaments in non-muscle cells is obtained due to the reaction between antibodies and intermediate filament-specific protein(s) other than actin. Actin antibodies do not react with intermediate filaments. At first sight these results might suggest that there is no actin in the intermediate filaments. However, to obtain fluorescence, the antibodies need to react only with some superficial protein components

of intermediate filaments. Thus, in an intermediate filament, if a central core of F-actin is hidden by intermediate filament-specific accessory proteins, actin antibodies would not be expected to gain access to the central F-actin core. Hence it is not surprising that the characteristic immunofluorescence pattern of intermediate filaments is obtained only with antibodies raised against intermediate filament-specific proteins, not against actin. Accordingly, the results obtained from immunofluorescence studies may be reconciled with the results described in this chapter.

Although HMM labelling is the best method available to identify F-actin (Huxley, 1963), caution should be observed in concluding that all filaments which exhibit HMM labelling existed before HMM treatment. This is because HMM is known to induce polymerization of G-actin into filaments (Yagi *et al.*, 1965). These filaments could then decorate with HMM. While unbound G-actin would be washed out of the cells by the procedure of glycerination, myosin-extraction (which involves several washes), there may still be some tiny quantities of monomeric or short oligomeric actin present within the cells. The only way to overcome this problem, as suggested by Huxley (1973), is to establish whether labelled filaments present in HMM-treated cells occur in the same regions and in similar numbers in non-HMM treated control cells. This was found to be true in the present studies. Accordingly, it can be argued that it is extremely unlikely that the filaments which were interpreted as labelled intermediate filaments



are simply the products of HMM-induced polymerization of G-actin.

A role for intermediate filaments in organelle movement has been suggested because of the association between intermediate filaments and organelles such as mitochondria (Starger and Goldman, 1977). If the mechanical force for organelle movement is based on an actomyosin system, then a core of F-actin within intermediate filaments could be functionally meaningful (Buckley, Raju and Stewart, 1978). The accessory proteins could be involved in regulating the actomyosin interaction by blocking and unblocking the myosin binding sites on actin filaments.

Another way in which actin within intermediate filaments might be functionally meaningful is in the maintenance of the three dimensional shape of the cell. Although it has long been recognized that much filamentous actin occurs in the cell cortex (Ishikawa, Bischoff and Holtzer, 1969), it has been assumed that relatively little exists in the deeper cytoplasm. The results presented above, indicating a core of F-actin within many sub-cortical filaments, support the view (Pollard, 1976a) that there may exist, throughout the cytoplasm of some cells, a supporting actin-based network which is important for cell shape determination.

Future work has to be done in the following areas to investigate the possibility that F-actin forms the basis of the intermediate filaments in some other non-muscle cells:

- (1) Determining the effect of gentle trypsin-treatment on intermediate filaments biochemically, using SDS polyacrylamide gel electrophoresis of cells with and without trypsin treatment;
- (2) Carrying out immunofluorescence staining of trypsin-treated cells using actin-specific antibodies to determine whether such treatment could result in the staining of intermediate filaments;
- (3) Using techniques other than HMM labelling to demonstrate that F-actin is present in intermediate filaments (see next chapter);
- (4) Demonstrating by in vitro model experiments how the intermediate filament-specific proteins of various cell types can associate with F-actin to form intermediate filaments and, in so doing, block HMM binding;
- (5) Investigating the nature of intermediate filaments in many other types of cells using the modified HMM labelling technique described in this chapter.

## CHAPTER 5 - IDENTIFICATION AND LOCALIZATION OF F-ACTIN IN CYTOPLASMIC FILAMENTS:

### II. STUDIES USING DNAase I

## INTRODUCTION

HMM labelling of filaments in cultured chick embryo connective tissue cells under various conditions, as described in the previous chapter, has indicated that most of the 4-11 nm diameter filaments which permeate the cytoplasm, either in bundles or networks, contain actin. To gather collateral evidence which might support this finding, it was decided to apply a technique other than HMM labelling to localize actin.

A survey of the relevant literature revealed that bovine pancreatic deoxyribonuclease I (DNAase I) depolymerizes muscle F-actin in a highly selective manner (Hitchcock, Carlsson and Lindberg, 1975 and 1976; Mannherz *et al.* 1975). DNAase I, an endonuclease, was first isolated and crystallized from bovine pancreas by Kunitz in 1948 (Kunitz, 1950). It is a glycoprotein with a molecular weight of 31,000 daltons. As early as 1949 it was known that eukaryotic cells contain a protein that strongly inhibits DNAase I (Dabrowska, Cooper and Laskowski, 1949; Cooper, Trautmann and Laskowski, 1950). Subsequently, it was found that the primary structure of this inhibitor was closely related to muscle G-actin (Lazarides and Lindberg, 1974). The extremely tight binding of G-actin to DNAase I ( $K_a$  of inhibitor for DNAase I is about  $10^{10} M^{-1}$  (Berger and May, 1967)) argues that the interaction between G-actin and DNAase I is highly specific.



Later Hitchcock, Carlsson and Lindberg (1976) found that F-actin can also interact with DNAase I, resulting in the inhibition of DNAase I activity and the depolymerization of the F-actin. These authors also analysed the DNAase I: F-actin interaction by electron microscopy and found that there was an initial thickening of filaments, followed by the formation of lateral protuberances. They also noticed the presence of shorter filaments suggesting that fragmentation might occur as part of the depolymerization process. Two hours after the addition of DNAase I to F-actin, when the depolymerization of F-actin was almost completed (as determined by viscometry), there was a background of globular protein, with occasional large aggregates which the authors interpreted as agglomerated G-actin:DNAase I complexes. From this experiment, it was concluded that DNAase I causes depolymerization of actin filaments by attacking over their entire length rather than only at their ends. It should be stressed here that the depolymerization of F-actin is caused not by any enzymic action of DNAase I but that it is due to stereo-specific binding between each DNAase I molecule and each G-actin monomer within the F-actin filament, this resulting in the breakage of inter-monomeric links. Regulatory proteins such as troponin and tropomyosin, which are normally associated with F-actin in skeletal muscle, slow down but do not prevent this in vitro depolymerization of F-actin (Hitchcock, Carlsson and Lindberg, 1976).

Stimulated by these findings, it was decided to see whether DNAase I would also interact with actin filaments

in non-muscle cells to bring about their depolymerization. Cells used for this purpose were glycerinated and myosin-extracted so that they would be permeable to DNAase I. It was already known that such extraction procedures preserved the 4-11 nm filaments in the bundles and networks (Buckley and Raju, 1976).

Before attempting to study the effect of DNAase I on filaments within the cell, it was decided to do experiments similar to those done by Hitchcock, Carlsson and Lindberg (1976) on muscle F-actin:

- (i) to study the fine structural stages involved in the depolymerization of muscle F-actin,
- (ii) to determine the morphological differences, if any, between the depolymerization stages of single F-actin filaments and those of  $Mg^{2+}$  paracrystals of F-actin, and
- (iii) to establish that the batch of DNAase I to be used in the cell experiments was effective in depolymerizing filaments of muscle F-actin.

The use of DNAase I as a probe to localize actin filaments in cultured cells was first shown by Raju, Stewart and Buckley (1978) who demonstrated the removal of filaments in the sub-plasmalemmal region, i.e. filaments already widely believed to contain actin on the basis of HMM decoration experiments (Allison, Davies and De Petris, 1971). However, once the success of the application of this

method to cells was established, further experiments were carried out to show that DNAase I can be used as an effective tool to identify actin-containing filaments in other regions of the cytoplasm as well. It should be stressed that when this technique is carried out as an adjunct to HMM binding experiments, the combined evidence becomes more compelling.

The results in this chapter are grouped as follows:

- (a) Ultrastructure of negatively stained F-actin with and without DNAase I treatment;
- (b) Ultrastructure of  $Mg^{2+}$  paracrystals of actin with and without DNAase I treatment;
- (c) DNAase I action on sub-plasmalemmal filaments of glycerinated, myosin-extracted cells;
- (d) DNAase I action on cortical and sub-cortical (deeper) filaments;
- (e) SDS polyacrylamide gel electrophoretic analysis of the DNAase I extract.

## RESULTS

(a) *Ultrastructure of negatively stained F-actin with and without DNAase I treatment:* F-actin was treated with DNAase I for 2 hours at room temperature (for details, see Chapter 2, P. 44). Careful controls were carried out in conjunction with all these F-actin:DNAase I experiments to determine whether the structure of F-actin is altered simply by standing at room temperature. For this, a sample from the same batch of F-actin used in the DNAase I

experiments was kept for 2 hours at room temperature. At the end of this period it was prepared for electron microscopy by negative staining. Electron micrographs of this material show elongate filaments,  $\sim 6$  nm in diameter, which have the characteristic substructure of F-actin (Fig. 1). This finding indicates that the actin structure is not affected by temperature alone.

By contrast, when F-actin was treated with DNAase I for 2 hours at room temperature, the actin filaments were depolymerized in a way similar to that reported by Hitchcock, Carlsson and Lindberg, (1976). Here, one can describe four different stages in the depolymerization of F-actin. These stages are as follows:

- (i) In the first stage, even though the filaments remain intact, their surfaces appear rough, as if due to the attachment of tiny globules of material. This attachment seems to be regular in some areas but not so in others (Fig. 2). The occasional periodicity of attachment can be distinguished readily from the arrowhead formations obtained after HMM treatment.
- (ii) In the next stage the filaments appear to be swollen and noticeably variable in diameter (Fig. 3).
- (iii) In the third stage (Fig. 4), the swollen filaments show more pronounced lateral protuberances. Subsequently the swollen filaments begin to break up and large



Fig. 1: Electron micrograph of a negatively stained preparation of purified skeletal muscle F-actin showing long 5-7 nm diameter filaments. As a control for the DNAase I experiment, this particular preparation of F-actin was left standing at room temperature for 2 hours. As illustrated in the micrograph, this procedure had no discernible effect on the structure of actin filaments. X 126,000.

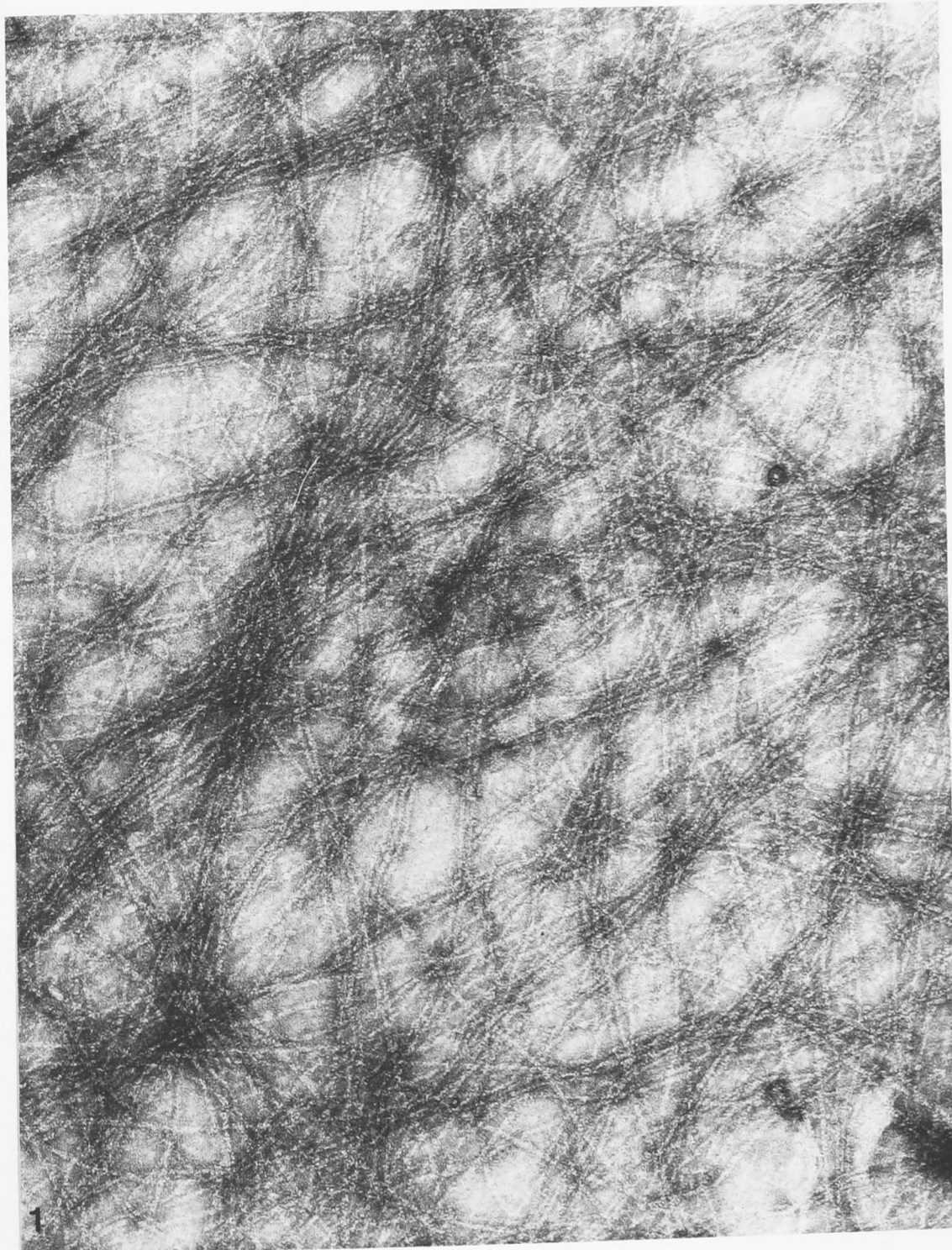


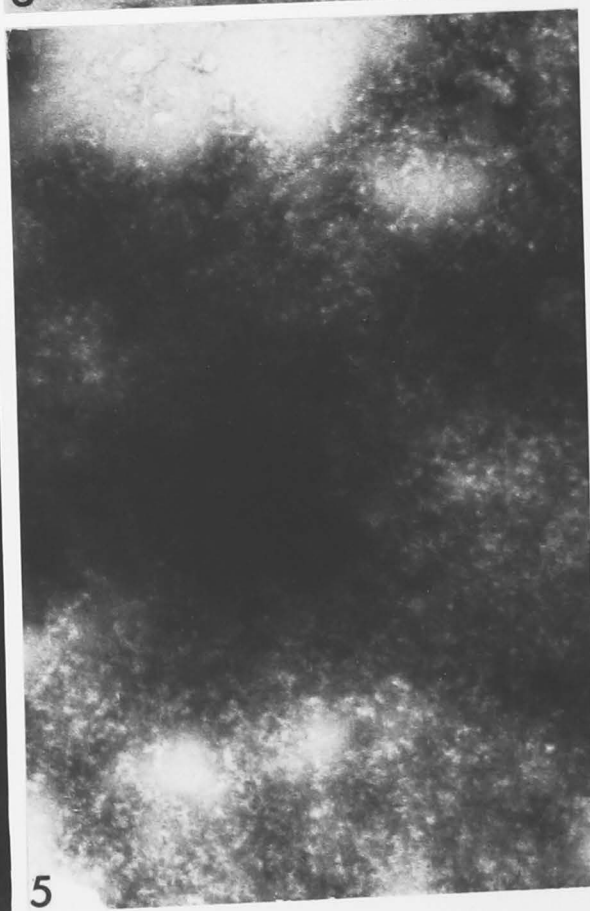
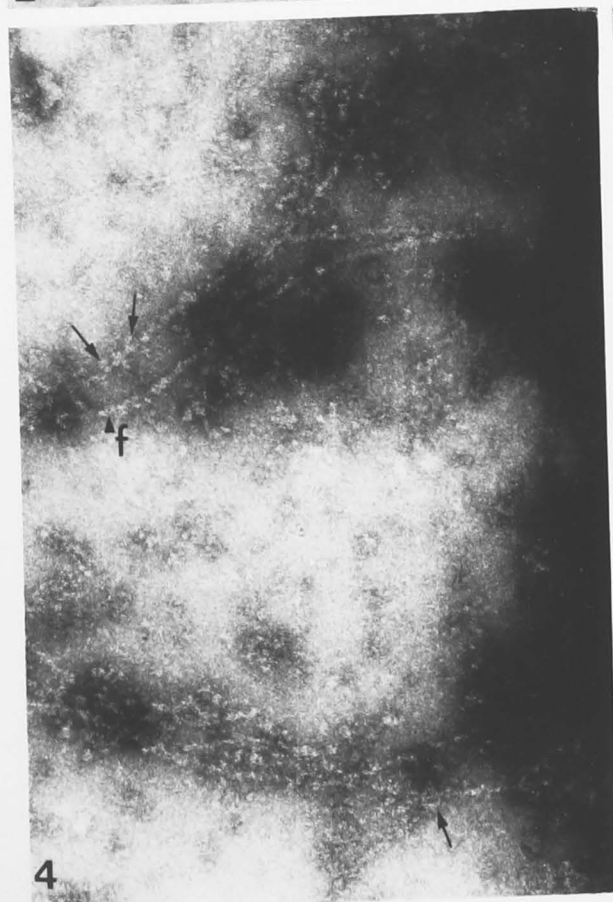
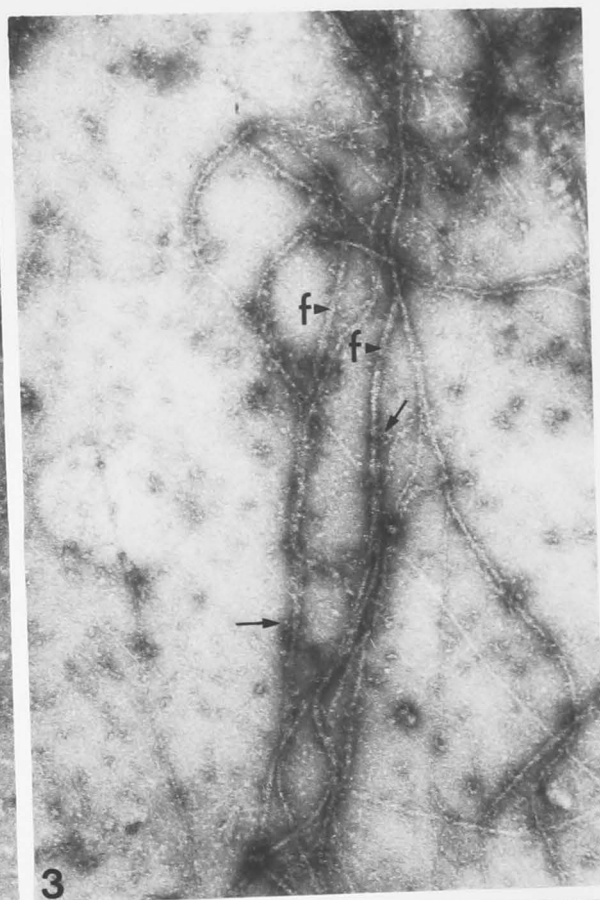
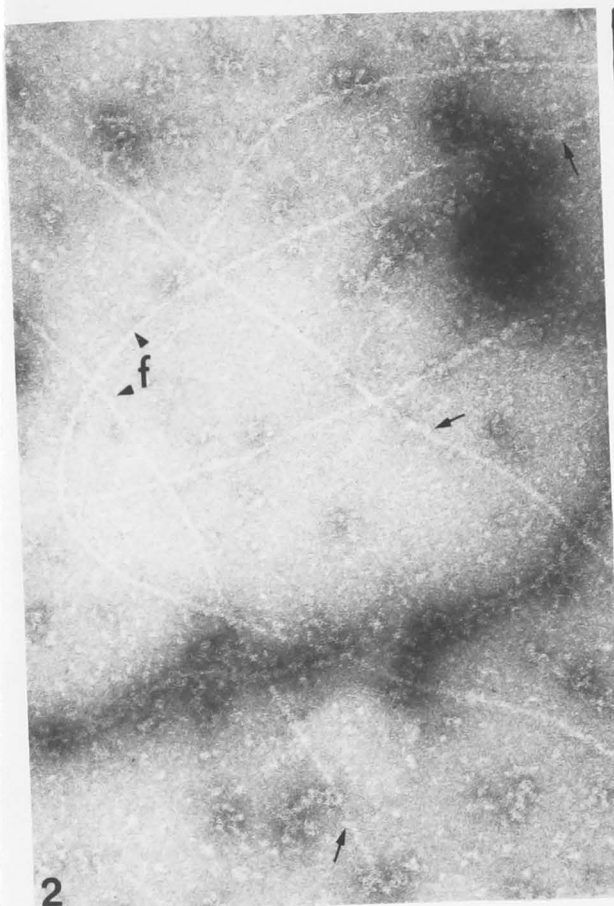
Fig. 2: Electron micrograph of a negatively stained preparation of F-actin which has been treated with DNAase I for 2 hours at room temperature (for methods, see Chapter 2, p.44). Several long filaments (f) are seen. The surface of some filaments appears rough, as if due to the attachment of some globular material (arrows). In the same field there is a short filament which looks completely different from the surrounding filaments by its heavy decoration with amorphous material. X 70,000.

Fig. 3: Electron micrograph showing F-actin filaments which were treated with DNAase I in the same fashion as those shown in Fig. 2. Most of the filaments (f) in this field appear swollen and have associated with them globular excrescences (arrows). However, there are a few filaments which are thin and resemble those in Fig. 2. X 70,000.

Fig. 4: Electron micrograph of a negatively stained F-actin-DNAase I preparation showing a swollen actin filament (f) exhibiting lateral globular protuberances (arrows). The filament is no longer continuous. Towards one end, the filament structure is completely lost and a large electron-dense aggregate is formed. X 70,000.

Fig. 5: In this figure no filaments can be observed. Only a large, densely staining, complex of aggregated globular material is present. X 70,000.







amorphous aggregates are formed, this leading to the next stage.

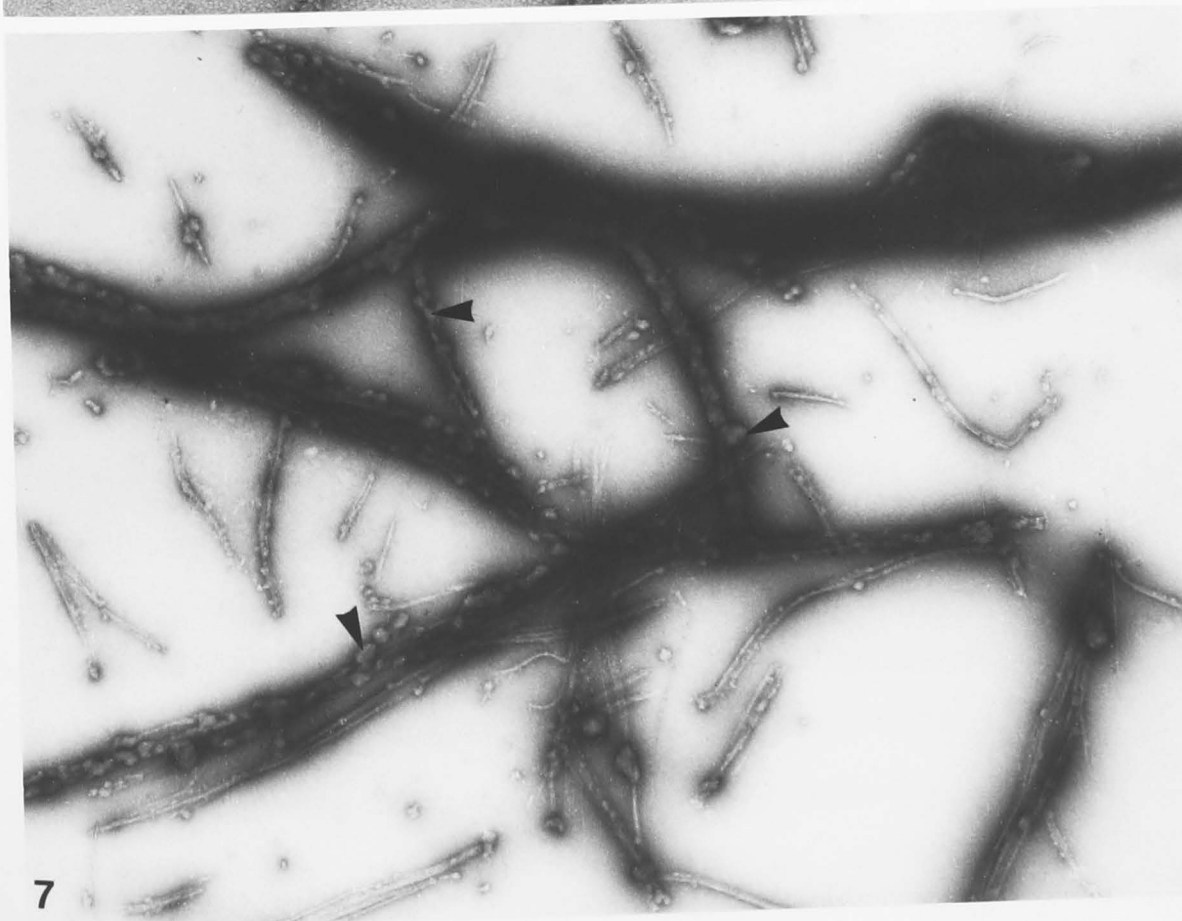
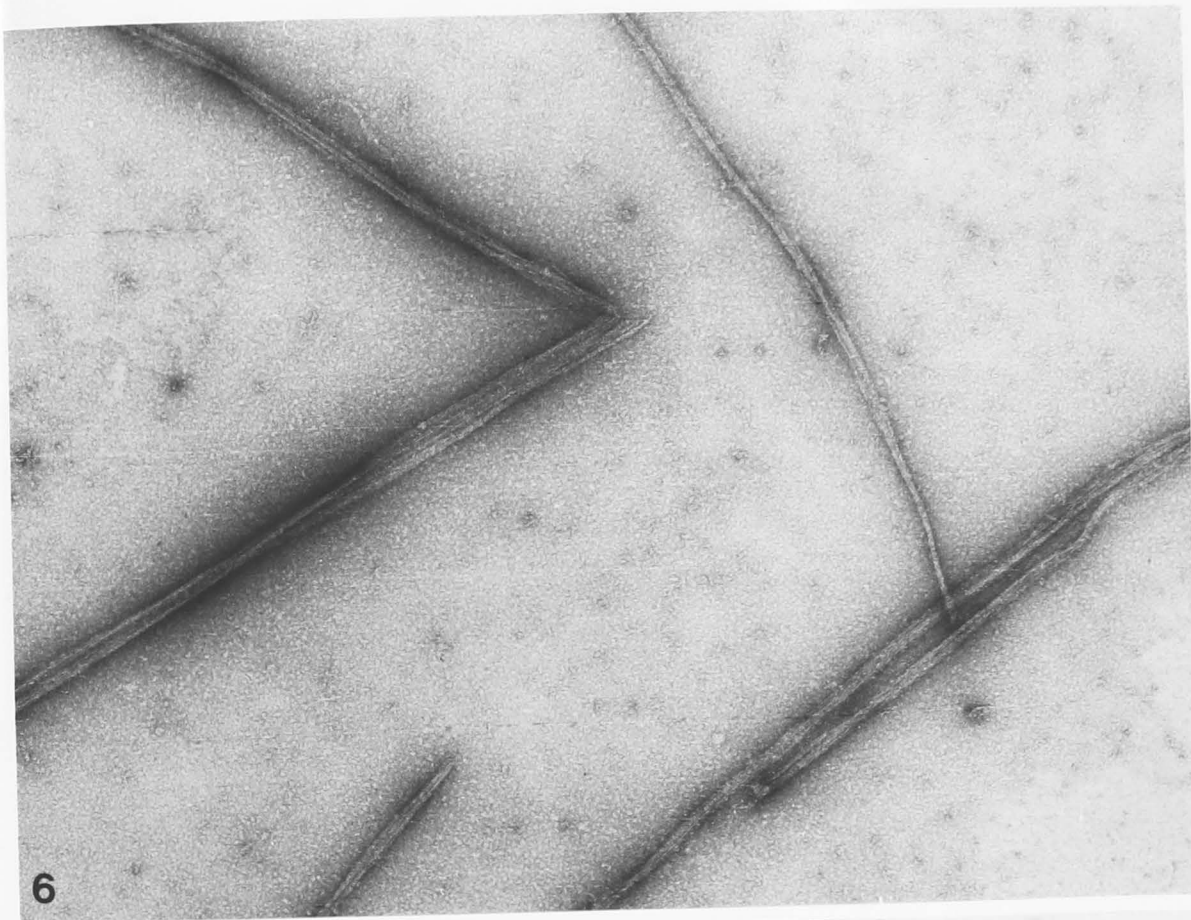
- (iv) Finally, in the fourth stage (Fig. 5) the filamentous nature is completely lost and irregular masses of globular material, presumably made up of G-actin:DNAase I complexes (Hitchcock, Carlsson and Lindberg, 1976) are seen.

For reasons which are not completely understood, it is noteworthy that all of these four stages may often be seen within a single preparation.

(b) *Ultrastructure of  $Mg^{2+}$  paracrystals of actin with and without DNAase I treatment:* As with the F-actin control, in the absence of DNAase I, the structure of  $Mg^{2+}$  paracrystals of actin is not altered simply by standing at room temperature for 2 hours (Fig. 6). By contrast, when incubated in the presence of DNAase I for 2 hours at room temperature, the structure of the paracrystals is grossly altered and the constituent filaments become completely covered with globular aggregates (Fig. 7). However, complete loss of structure (which was witnessed in some instances in the case of single F-actin filaments after treatment with DNAase I for 2 hours) did not occur in the case of the  $Mg^{2+}$  paracrystals. Indeed it took two to three times as long for  $Mg^{2+}$  paracrystals to undergo a comparable degree of depolymerization than it did for the single F-actin filaments, when both were treated with DNAase I simultaneously.

Fig. 6:  $Mg^{2+}$  paracrystals of F-actin which have been negatively stained after being kept at room temperature for 2 hours as a control for DNAase I action on  $Mg^{2+}$  paracrystals. As the micrograph shows, the paracrystal structure of actin is not affected by this standing at room temperature. The filaments, which occur tightly packed within the paracrystals, do not show any lateral protuberances and are not covered by any globular material. X 45,000.

Fig. 7: After 2 hours of treatment with DNAase I at room temperature, the paracrystals are completely covered with globular aggregates (arrowheads). In some instances, the paracrystals are broken into smaller fragments and the filaments within the paracrystals are no longer identifiable. X 50,000.



(c) *DNAase I action on sub-plasmalemmal filaments of glycerinated, myosin-extracted cells:* As mentioned in the introduction to this chapter, the action of DNAase I on sub-plasmalemmal filaments was first examined in relation to developing a method for the selective extraction of actin-containing filaments (Raju, Stewart and Buckley, 1978). Fig. 8 shows a typical view of the sub-plasmalemmal region of a control cell, i.e. one which has been glycerinated and myosin-extracted but not treated with DNAase I. Large numbers of thin (4-7 nm) filaments occupy the veil-like extensions of the cell margin and, except where gathered into narrow bundles which occupy the cores of microvilli, the arrangement of these filaments is essentially random, so that they form a loose meshwork. As one moves deeper into the cytoplasm, some filaments are seen in the form of elongate bundles. Such filaments, within the veil-like extensions of cells are known to bind HMM, indicating that they contain actin (Allison, Davies and De Petris, 1971; see also previous chapter).

After treatment with DNAase I for 2 hours at room temperature and overnight at 4°C (for method, see Chapter 2, p.35), the earliest and most striking change within the veil involves the loose meshwork of sub-plasmalemmal filaments. These disappear almost completely (Fig. 9). Filaments which were gathered into microvillus bundles take longer to depolymerize. At this early stage the filaments in the bundles are characterized by the presence on their surfaces of amorphous protuberances which closely resemble those observed on actin filaments at Stage (iii) of their depolymerization after treatment with DNAase I (Fig. 4).



Fig. 8: Electron micrograph of the sub-plasmalemmal region of a fixed and negatively stained cell which has been glycerinated and myosin-extracted before fixation (for methods, see Chapter 2, p.32). The cell was not treated with DNAase I and thus serves as a control for the DNAase I-treated cell. Large numbers of 4-7 nm diameter filaments (f) occupy the veil-like extension (v) of the cell margin. The arrangement of filaments is essentially random, forming a loose meshwork, except where gathered into bundles which occupy the core of a microvillus (mv). Deeper to the veil, some filaments are arranged in the form of elongate bundles (fb). X 70,000.

Fig. 9: Electron micrograph of the sub-plasmalemmal region of a cell after treatment with DNAase I (for Methods, see Chapter 2, p.32). Note the complete absence of the loose meshwork of filaments in the veil (v). Narrow bundles at this stage are transformed into linear aggregates of amorphous material with excrescences (arrows) which have developed on the filaments. X 70,000

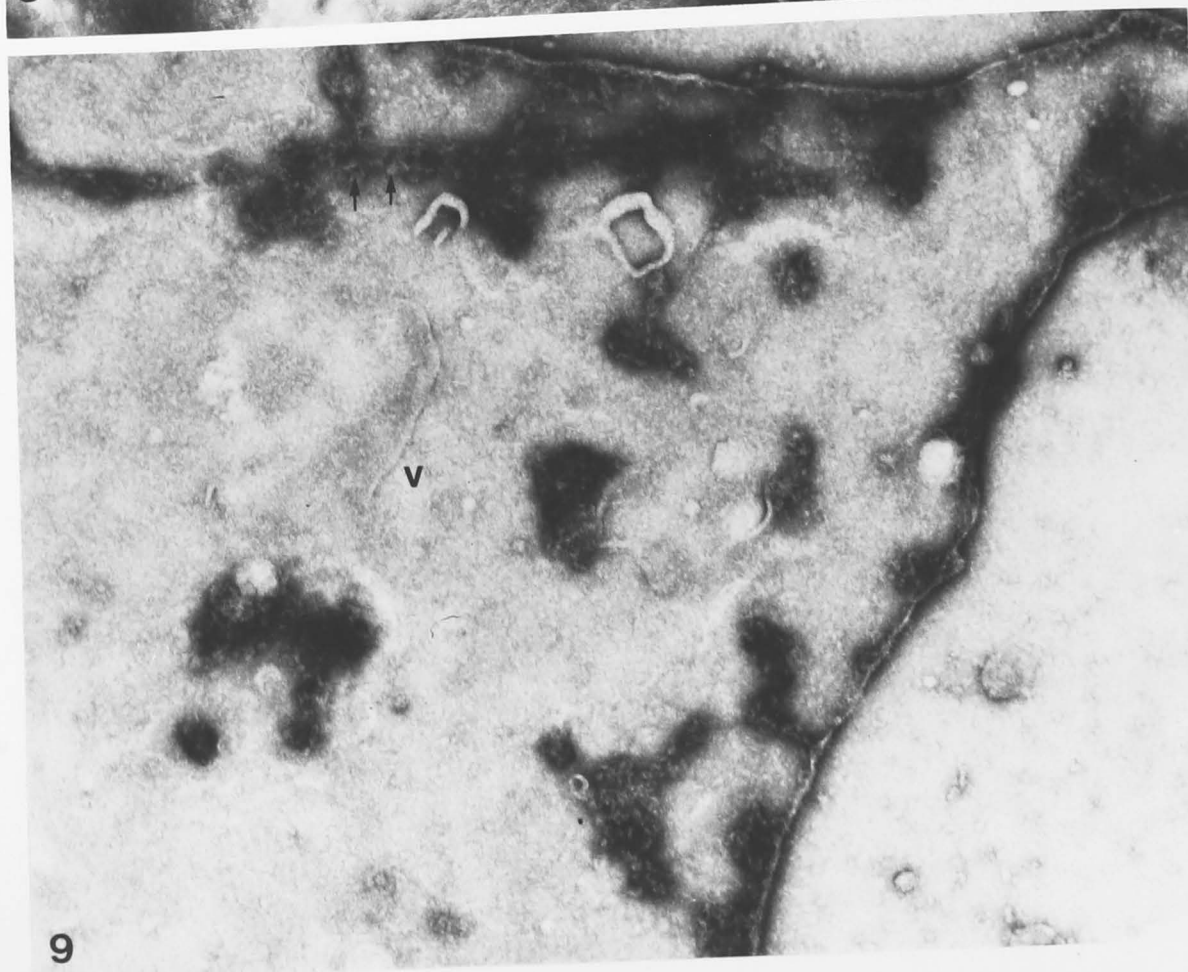
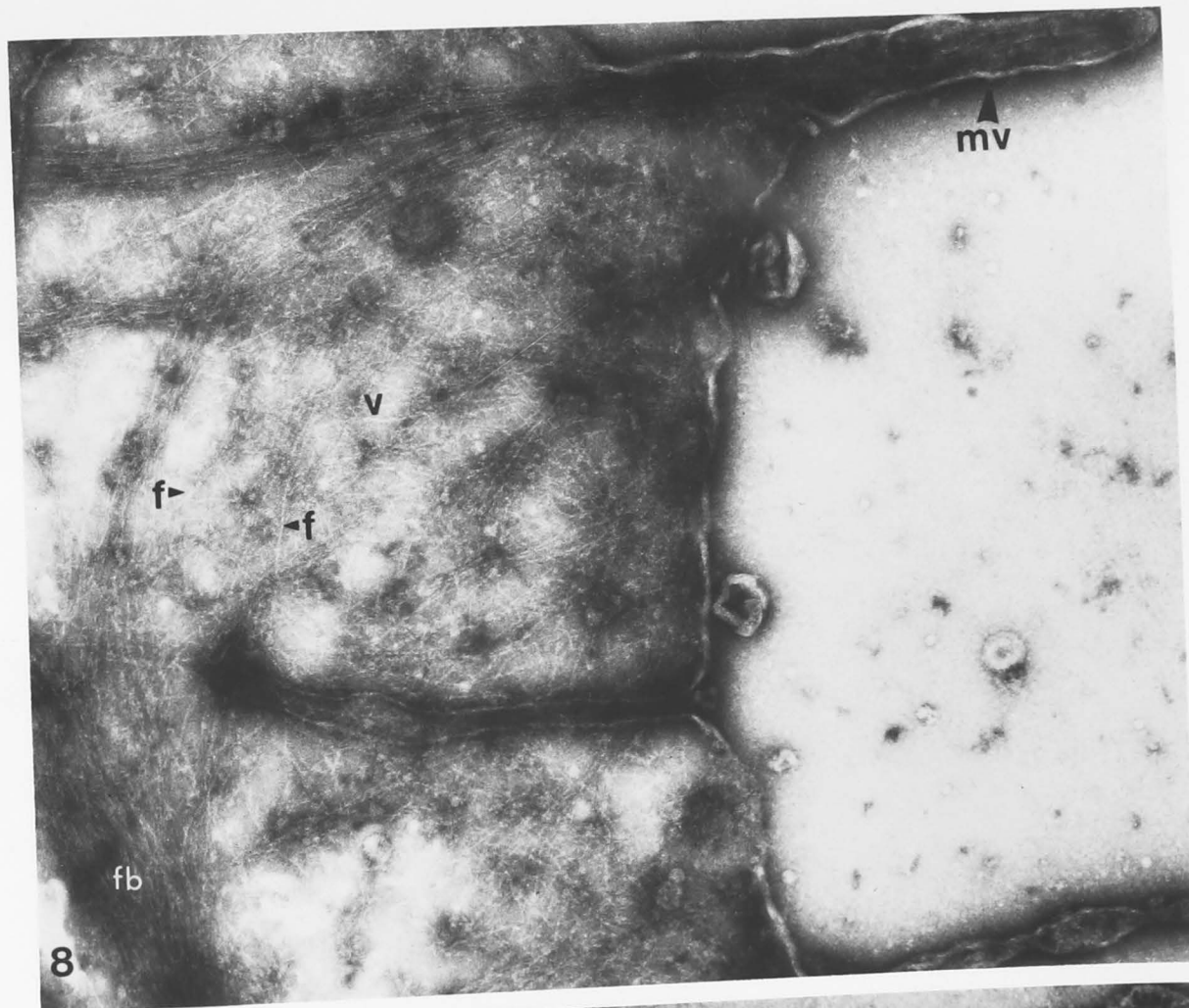


Fig. 10 is also a DNAase I treated cell, which shows the absence of most of the sub-plasmalemmal filaments but the few which remain are increased in diameter in an irregular fashion, indicating the likelihood that initial swelling of filaments similar to that occurring in muscle F-actin during the process of depolymerization may also occur in these sub-plasmalemmal filaments.

(d) *DNAase I action on cortical and sub-cortical (deeper) filaments:* Filaments in the cortical region are arranged in parallel, in the form of long bundles and sheets (Buckley and Porter, 1967; Buckley, 1975; see also Fig. 8). These filaments are 4-7 nm in diameter and are known to decorate with HMM, indicating that they contain actin (Ishikawa, Bischoff and Holtzer, 1969). When treated with DNAase I, although the bundles have not completely disappeared, many filaments within the bundles are completely transformed to a series of linear aggregates of amorphous material (Fig. 11). In some cells the bundles have completely disappeared.

The great majority of filaments in the sub-cortical or deeper cytoplasm is of 'intermediate-size' (7-11 nm) (Ishikawa, Bischoff and Holtzer, 1969; Goldman and Knipe, 1973). When treated with DNAase I, all the sub-cortical filaments disappeared completely (Fig. 11). Because there is much independent evidence showing the actin nature of filaments in the sub-plasmalemmal region and in the cortical bundles, the results obtained for those filaments were expected, but a similar interpretation for the sub-cortical intermediate filaments, which were believed

Fig. 10: Sub-plasmalemmal region of another cell treated similarly to the one shown in Fig. 9, illustrating the absence of most of the filaments in the veil (v). The few filaments (f) which are present are swollen in diameter ( $\sim 15$  nm) and appear similar to the DNAase I-treated actin filaments shown in Fig. 3. Dense staining complexes (c) are also seen in this region. X 70,000.



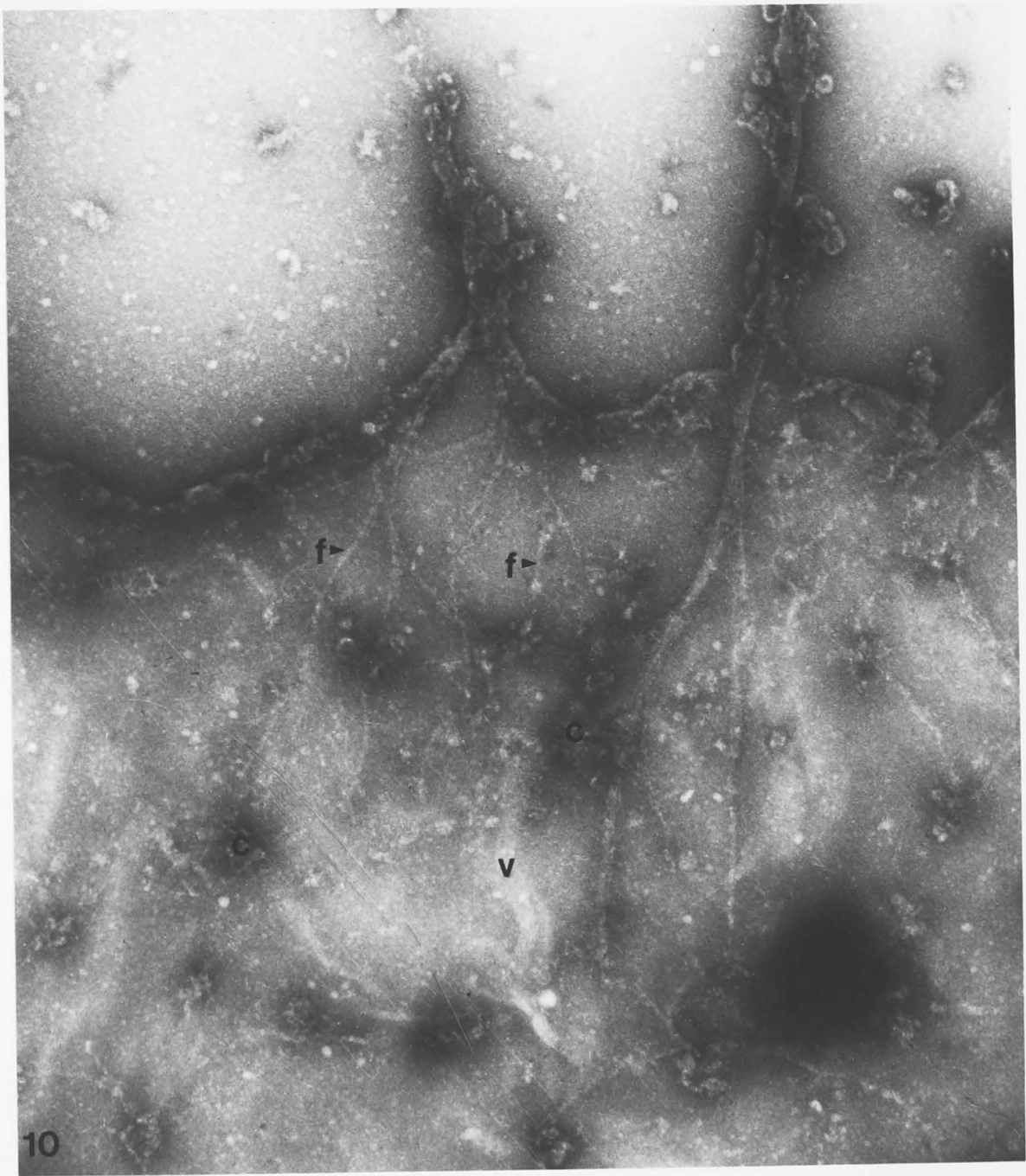


Fig. 11: A portion of a glycerinated and myosin-extracted cell treated with DNAase I (using the same procedure as used in the cells depicted in Figs. 9 and 10). The filaments in the veil (v) and sub-cortical (sc) regions have all disappeared. However, the cortical filament bundles (fb) have not disappeared completely. Nevertheless, the filaments within the bundles are neither continuous nor readily identifiable as filaments. Only linear aggregates of globular material are seen. X 70,000.

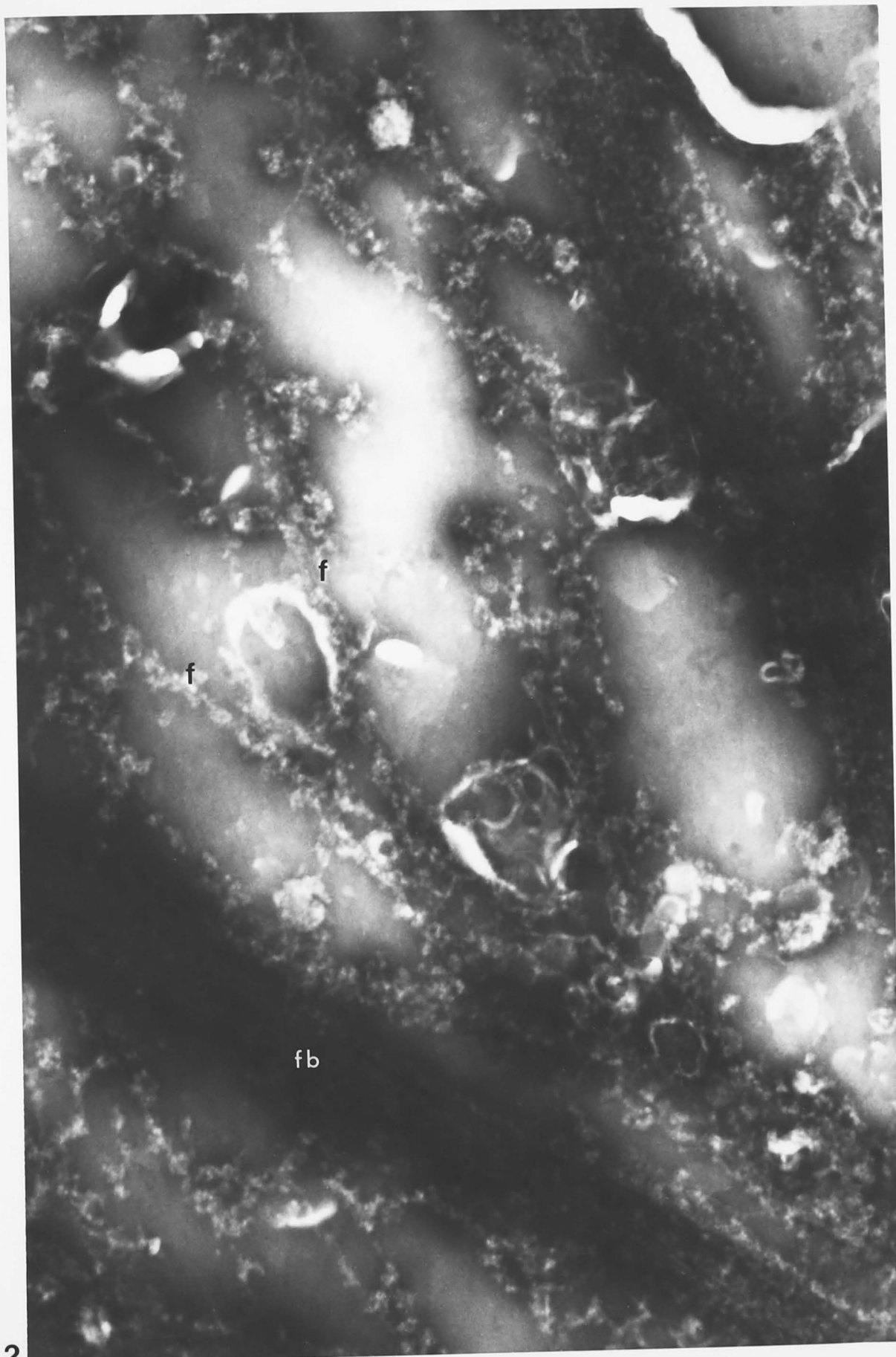


to be non-actin in nature (Ishikawa, Bischoff and Holtzer, 1969; Starger and Goldman, 1977) had to be questioned. Therefore, it was asked whether intermediate-sized filaments were lost due to depolymerization or whether they were simply washed out of the cells as intact filaments after losing their attachment with membranes (which could be through associated actin filaments). If it could be shown that the intermediate filaments were lost by depolymerization then (because of the known selectivity of DNAase I for actin), it could be safely concluded that intermediate filaments too contain F-actin.

In order to find out which interpretation was correct, the cells were treated with DNAase I for a shorter period, i.e. for 2 hours at room temperature (cf. normal duration of DNAase I treatment of cells which is 2 hours at room temperature and 24 hours at 4°C), during which time even sub-plasmalemmal filaments, which are known to contain actin (see above), are not completely lost but show amorphous excrescences throughout their length. If the sub-cortical filaments (which are mostly of intermediate size) do not contain actin, that would be indicated not only by their continued presence but also by their freedom from changes characteristic of the action of DNAase I on actin. To illustrate the result, Fig. 12 shows a portion of a glycerinated, myosin-extracted cell which has been treated with DNAase I for 2 hours at room temperature. Here it can be clearly seen that not only are the filaments within the bundles affected but so also are the sub-cortical filaments. Some sub-cortical filaments show lateral



Fig. 12: Electron micrograph of a portion of a cell where the duration of DNAase I treatment was limited to 2 hours at room temperature. The filaments within the bundles (fb) are disorganized, appearing as linear aggregates of amorphous material. The interesting feature here is that even the sub-cortical filaments (f) are still present, though also transformed to aggregates of amorphous material. X 52,000.

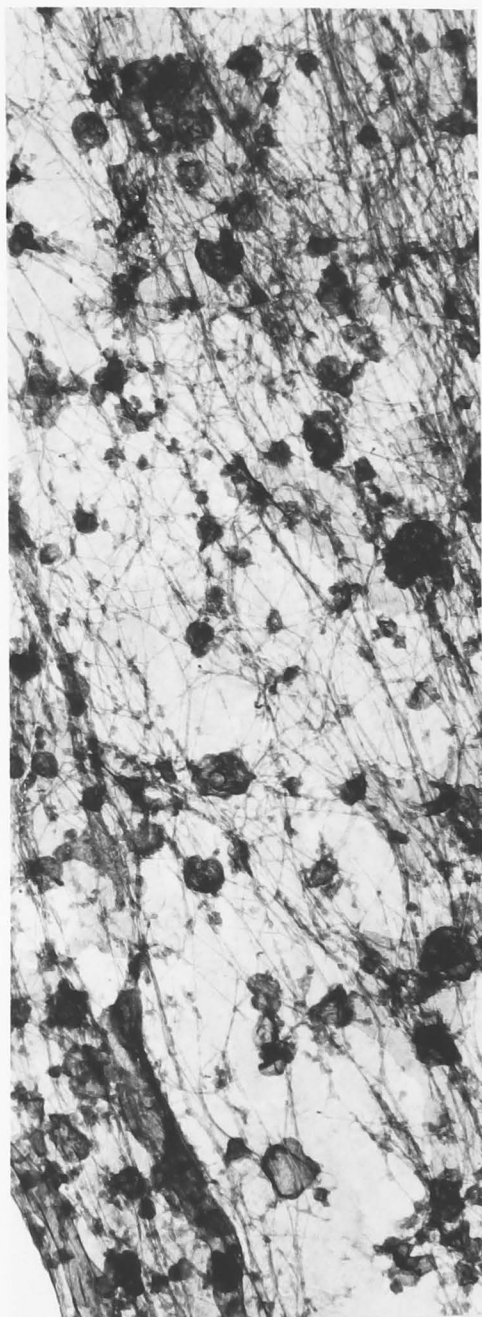


protuberances while others are transformed into linear aggregates of amorphous material, similar to those seen with muscle actin filaments treated with DNAase I. Moreover, at later stages, where complete loss of intermediate filaments from the cells had occurred, no intermediate filaments were observed extracellularly, adhering to the hydrophilic surface of the formvar/carbon-coated grids. This observation makes it seem unlikely that entire intermediate filaments could have disappeared simply by being washed out of the cell.

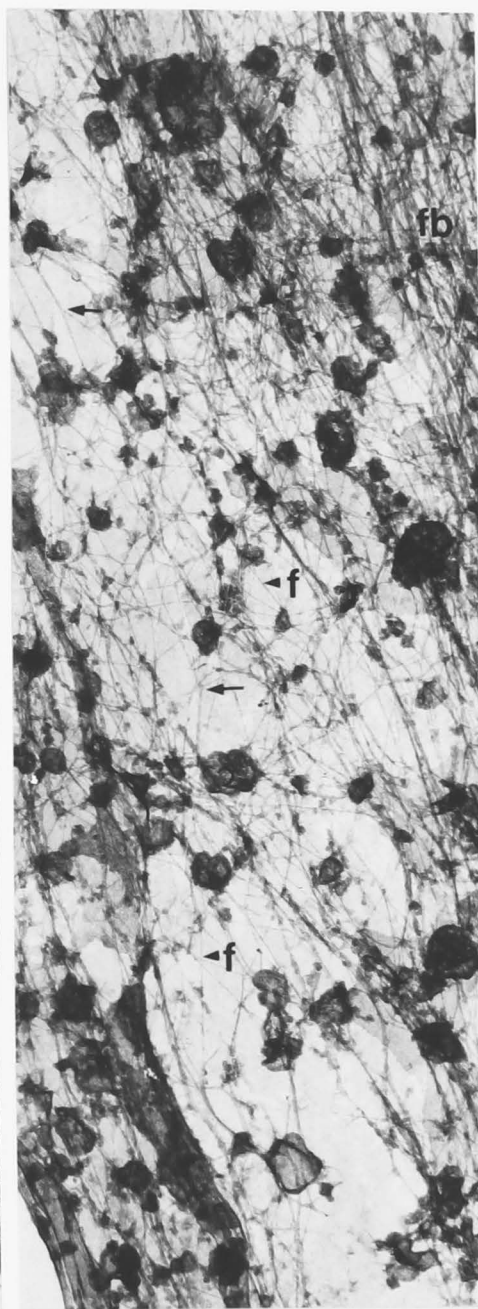
DNAase I-treated cells were also fixed and critical-point dried (CPD) to see whether results obtained with negative staining could be due to some artefact of the staining procedure. As illustrated in Figs. 13a and b, glycerinated, myosin-extracted cells show a well defined three-dimensional network of filaments and filaments bundles. After DNAase I treatment, at late stages, the filaments are completely lost (Fig. 14) and, at earlier stages, where the filaments are not completely lost, they are decorated with globular excrescences, similar to those seen in negatively stained preparations (Figs. 15a and b). In such preparations the remnants of filaments are commonly seen to end abruptly. Amorphous aggregates are also present within the cell. The stereo pair of micrographs (Figs. 15a and b) also show that the enveloping cell membrane is well preserved, this indicating the likelihood that filaments are not lost due to any overall loss of cell membrane together with filamentous contents.

Figs. 13a and b: Stereo pair of electron micrographs of a cell which has been fixed and CPD after myosin extraction. The cell shows a three dimensional network of filaments (f). The filaments forming the network range from 4-11 nm in diameter and show true branching (arrows). A loose bundle of filaments (fb) is also present. X 44,100.



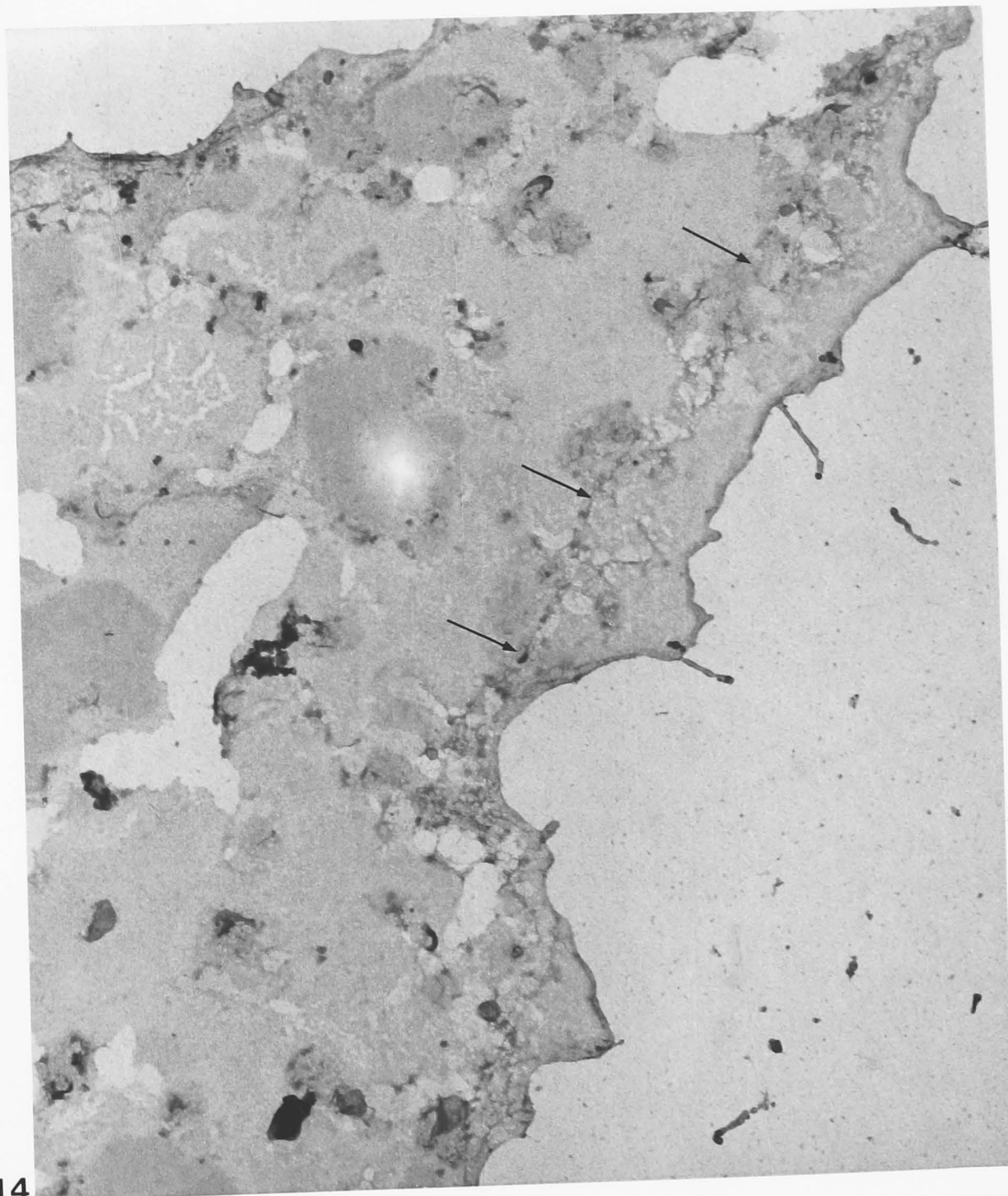


13a



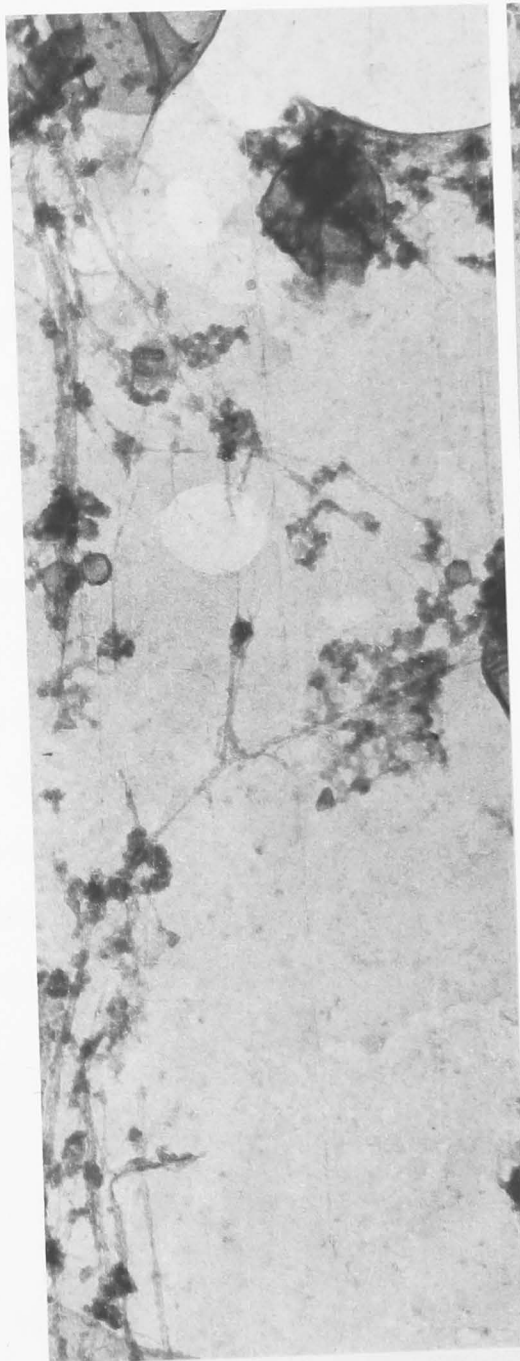
13b

Fig. 14: Electron micrograph of a glycerinated myosin-extracted cell which had been treated with DNAase I before fixation and CPD. Note the complete absence of filaments. There is a faint trace (arrows) of the sites where filament bundles were present before DNAase I treatment. X 44,100.

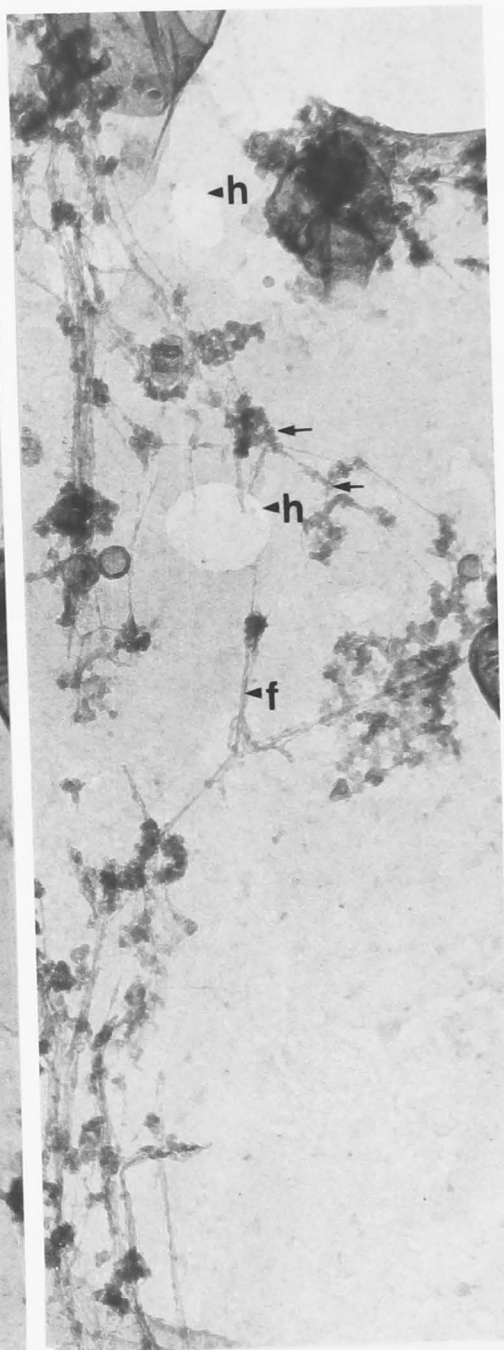


Figs. 15a and b: Stereo pair of electron micrographs of a portion of a cell which also received the same DNAase I treatment as the one in Fig. 14. The cell membrane is reasonably well preserved except for several holes (h). The few filaments (f) which remain within the envelope of membrane are decorated with some amorphous material (arrows). The filaments are not continuous, as shown by their abrupt endings. X 70,000.





15a



15b

(e) *SDS polyacrylamide gel electrophoretic analysis of the DNAase I extract:* The extract obtained after DNAase I treatment of the cells was applied to a gel (for details see Chapter 2, P.54). As Fig. 16 shows, there is a band present in the extract which comigrates with skeletal muscle actin, indicating that actin is extracted by this procedure. However, it can be noticed that apart from actin, there are distinct bands corresponding to the molecular weights of 40,000 daltons (40K), 31K (DNAase I), 23K and 13.5K. There are also some minor low molecular weight protein bands. To determine the origin of these bands, the enzyme preparation itself was analysed by electrophoresis. As Fig. 17 indicates, the major band corresponds to the molecular weight of DNAase I (31K). There are also distinct bands having molecular weights of 13.5K (RNAase) and 23K. The nature of 23K band and several other minor bands is not yet determined. It is assumed that these bands represent breakdown products of DNAase I. Work is in progress to explore this assumption.

### DISCUSSION

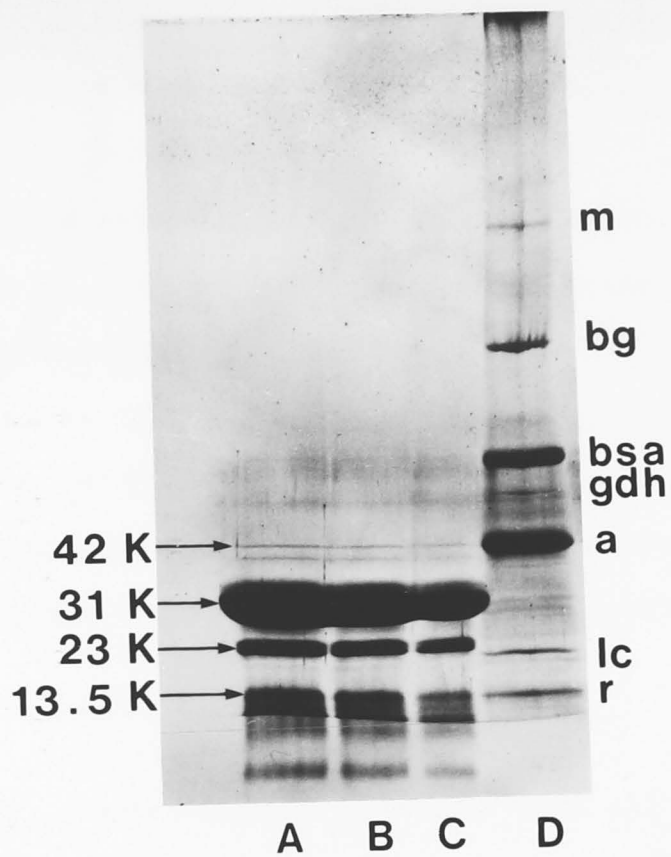
The results of the experiments described above can be summarized as follows:

1. Under the same conditions that DNAase I is known to depolymerize muscle F-actin, it depolymerizes not only the thin (4-7 nm) filaments, but also the intermediate-sized (7-11 nm) filaments of cultured chick embryo connective

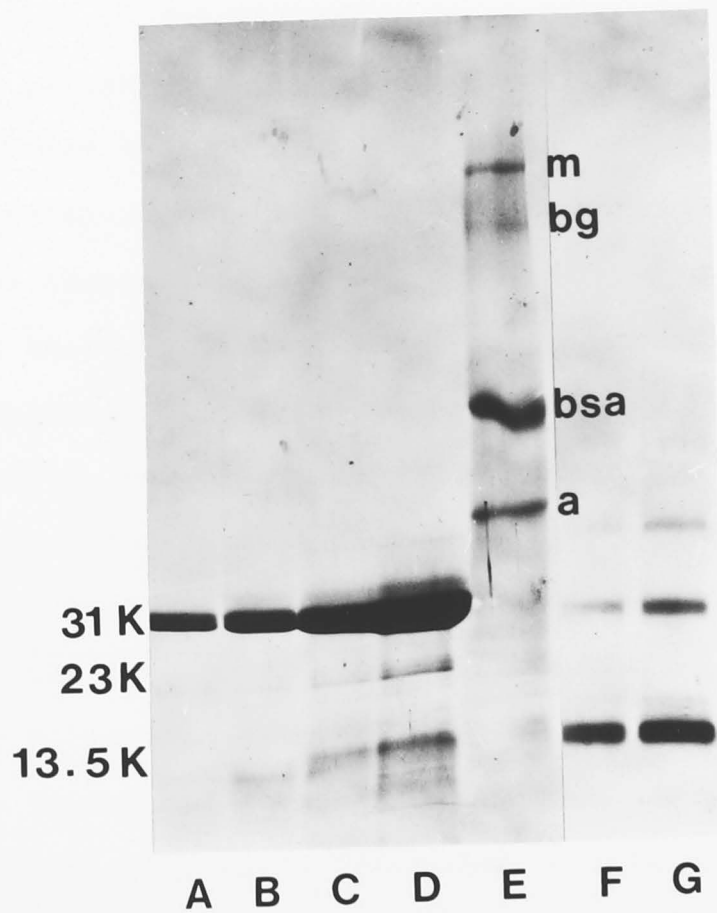
Fig. 16: SDS polyacrylamide gradient gel (3-15½%) electrophoretic profile (for methods, see Chapter 2, p.54) showing three different concentrations of the extract obtained after DNAase I treatment of cells (A,B,C). The extract shows the presence of a band comigrating with skeletal muscle actin (42K). A major band at 31K (DNAase I) and other distinct bands at 23K (unidentified) and 13.5K (RNAase) regions are also present. The origin of other minor bands, including the one in the 40K region, is not yet determined. The standards (D) consist of myosin heavy chain (m) (200K),  $\beta$ -galactosidase (bg) (130K), bovine serum albumin (bsa) (68K), glutamate dehydrogenase (gdh) (53K), skeletal muscle actin (a) (42K), myosin light chain (lc) (21K), and RNAase (r) (13.5K).

Fig. 17: SDS polyacrylamide gradient gel (6½-15%) electrophoretic profile of DNAase I enzyme preparation at 4 different concentrations (A,B,C,D). RNAase (F,G) of two different concentrations was also run along with DNAase I. As the figure indicates, the DNAase I sample, apart from a major band at 31K (which is due to DNAase I itself), has two distinct bands, one comigrating with RNAase (13.5K) and the other at the 23K region. There are also minor bands at 40K and in the low molecular weight ranges. The standards (E) consist of myosin heavy chain (m) (200K),  $\beta$ -galactosidase (bg) (130K), bovine serum albumin (bsa) (68K), and skeletal muscle actin (a) (42K).

16



17





tissue cells. The tightly packed filaments in the cortical bundles took a longer time to undergo depolymerization than the individually dispersed ones.

2. The morphologic appearances at various stages of the depolymerization of both muscle F-actin and these cytoplasmic filaments are essentially the same.

3. As determined by SDS polyacrylamide gel electrophoresis, the disappearance of filaments in the cytoplasm is accompanied by the appearance of a band comigrating with actin in the extract obtained by treating the cells with DNAase I.

Accordingly, these results indicate that all the filaments in the sub-plasmalemmal meshwork, in the cortical bundles and most of the filaments in the sub-cortical cytoplasm of glycerinated, myosin-extracted cells contain F-actin. These results are in complete agreement with the results obtained with the HMM binding experiments (see previous chapter and Buckley, Raju and Stewart, 1978).

Because their actin content is very well documented (Ishikawa, Bischoff and Holtzer, 1969; Goldman, 1975; Lazarides, 1976), the depolymerization and extraction of sub-plasmalemmal filaments, and filaments within the micro-filament bundles was not an unexpected result. In contrast, the depolymerization and extraction of intermediate filaments (7-11 nm in diameter) in the sub-cortical region may at first seem a surprising result. This observation contradicts the commonly held view that intermediate filaments do not contain actin (Small and

Sobieszek, 1977; Starger *et al.*, 1978). Hence these results warrant careful analysis. Before arriving at any firm conclusion, the following possibilities had to be considered:

- (a) Intermediate filaments could have been washed out of the cells as whole filaments either because they were attached to membrane through short actin filaments which underwent depolymerization when treated with DNAase I, or because they were lost through membrane disruption caused by glycerination;
- (b) A non-specific action of DNAase I itself, or some protease present in the DNAase as a contaminant, could have caused the destruction of intermediate filaments; and
- (c) Intermediate filaments do contain F-actin.

These possibilities will now be discussed.

(a) If intermediate filaments were washed out of the cell as whole filaments, it could be expected that at least some would be detected extracellularly, adhering to the hydrophilic surface of the formvar/carbon-coated grids. However, as indicated above, no filaments of any kind were observed extracellularly. The reasons that intermediate filaments could not have disappeared simply because of membrane disruption, which commonly occurs in glycerination, appear to be twofold. First, the controls, which were also subjected to glycerination, do show the presence of intermediate filaments. Secondly, as seen in some CPD cells, where the membrane is fairly well preserved (as shown by stereoscopic viewing) DNAase I treatment has, nevertheless, totally extracted intermediate filaments.

Moreover, whenever the cells were treated with DNAase I for only 2 hours at room temperature, neither the thin nor the intermediate filaments had disappeared completely. Instead these were transformed into linear aggregates of amorphous material showing lateral globular projections similar to those of an intermediate stage in the muscle F-actin depolymerization process. Accordingly, these observations argue against the possibilities that intermediate filaments had been removed from the cell either while still attached to membrane or as isolated but still intact filaments.

(b) The idea that the fragmentation and depolymerization of intermediate filaments by DNAase I might be due to some non-specific action of DNAase I or by a contaminating protease could be suggested by the claim that intermediate filaments are highly susceptible to proteases which have a trypsin-like action (Small and Sobieszek, 1977).

However, on the one hand, the highly selective action of DNAase I on actin (a protein known to be relatively trypsin-resistant (Tilney, 1977)), has been unequivocally demonstrated (Hitchcock, Carlsson and Lindberg, 1976); therefore it would seem highly improbable that DNAase I would, by some non-specific action, have caused the depolymerization of intermediate filaments, if these were made up exclusively of some protein other than actin.

On the other hand, it is highly unlikely that the depolymerization of intermediate filaments was caused simply by a protease contaminant in the DNAase I preparation: according to the manufacturer (SIGMA Chemical Company, St. Louis, Mo, USA), the preparation of DNAase I (Type:DNCL)

been indicated by RNA labelling experiments (see previous

used is chromatographically purified, the only contaminant being a trace of RNAase. Nevertheless, as shown in the gel (Fig. 17), there are bands other than DNAase I and RNAase. Accordingly, it can be argued that it is not possible to rule out the presence of proteases. However, strong evidence against the presence of proteases comes from the experiment involving the treatment of intermediate filaments isolated from chicken gizzard smooth muscle (see Chapter 7).

Chicken gizzard intermediate filaments are known for their extreme sensitivity to proteases (Small and Sobsieszek, 1977). Confirming this, it was found in this laboratory that a mild trypsin treatment, which does not destroy the intermediate filaments of cultured chick embryo connective tissue cells, completely destroys the intermediate filaments of chicken gizzard smooth muscle. However, the batch of DNAase I which depolymerizes the intermediate filaments of chick embryo connective tissue cells, has no action on intermediate filaments of chicken gizzard. Thus it can be concluded that DNAase I action on intermediate filaments of chick embryo connective tissue cells is a highly selective one due, specifically, to the DNAase I itself, and not due to any contaminating proteases.

(c) The other remaining possibility is that the 7-11 nm intermediate filaments of the sub-cortical cytoplasm have disappeared due to depolymerization caused by the specific actin-depolymerizing action of DNAase I. The presence of an F-actin core within the intermediate filaments has been indicated by HMM labelling experiments (see previous



chapter and Buckley, Raju and Stewart, 1978) and these results appear to support such a concept.

In the experiments described above on cells, the microfilament bundles took a longer time to undergo depolymerization than the individual free filaments. This could be due to steric hindrance factors, which may be explained by the very close proximity of filaments to one another and the presence of significant quantities of accessory proteins such as tropomyosin (Lazarides, 1976) within the filament bundles. As mentioned earlier, Hitchcock, Carlsson and Lindberg (1976), observed a decreased rate of depolymerization of actin filaments which were associated with tropomyosin and as reported in the results (See p.44) the magnesium paracrystals of actin took approximately two to three times longer than single actin filaments to undergo depolymerization. The free filaments in the cytoplasm also took a longer time to undergo depolymerization than the purified muscle actin filaments. This suggests the likelihood that these free cytoplasmic filaments are associated with accessory proteins. It may also be that there is a high level of accessory (actin-binding) proteins in the filament bundles.

The demonstration for the first time that DNAase I can depolymerize actin-containing filaments of non-muscle cells (Raju, Stewart and Buckley, 1978) and the results described in this chapter add support to the speculative physiological role of DNAase I in regulating the cytoplasmic levels of F-actin to meet motility function requirements (Hitchcock, Carlsson and Lindberg, 1976; Hitchcock, 1977; Korn, 1978). However, attempts to support this idea by

localizing DNAase I in cultured cells by biochemical and immunocytochemical techniques have so far, met with little success (Hitchcock, Carlsson and Lindberg, 1976).

In conclusion, it must be said that the use of DNAase I to localize actin filaments could be applied to different types of non-muscle cells (in conjunction with the HMM labelling technique) to obtain a complete picture of F-actin distribution. The following lines of investigation may prove to be worthwhile in establishing appropriate conditions for further experiments on other types of cells:

- (1) Investigations to determine the exact quantity of DNAase I to be used in each type of non-muscle cell to achieve depolymerization of actin-containing filaments;
- (2) Work to establish the time taken by DNAase I -
  - (i) to depolymerize cytoplasmic filaments completely so that total extraction of these filaments is possible, and
  - (ii) to cause partial depolymerization of filaments, so that filaments which are undergoing depolymerization can be localized within the cell and thus identified as actin-containing.

Future work must also be carried out to explain how DNAase I gains access to F-actin cores of intermediate filaments in cultured chick embryo connective tissue cells under conditions where HMM does not (see previous chapter and also Buckley, Raju and Stewart, 1978).

The possible significance of the presence of F-actin

in thin filaments as well as in intermediate filaments was discussed in the previous chapter.

CHAPTER 6 - IDENTIFICATION, LOCALIZATION AND FORM  
DETERMINATION OF CYTOPLASMIC MYOSIN;  
STUDIES USING MYOSIN-EXTRACTING SOLUTIONS  
AND MUSCLE CONTRACTILE PROTEIN MOLELS

As mentioned in the general introduction Chapter I, the occurrence of myosin in a wide variety of eukaryotic cell types is now well established (see Pollard, 1976; Pollard and Weisman, 1977; Clark and Osborn, 1977). The most convincing evidence comes from the biochemical isolation of myosin from non-muscle cells. Purified non-muscle myosins exhibit ATPase activity which is identical to that of skeletal myosin (see Pollard, 1976; Clark and Osborn, 1977; and Clark, 1978). Other evidence comes from electron microscopy (see Pollard, 1976).

CHAPTER 6 - IDENTIFICATION, LOCALIZATION AND FORM  
DETERMINATION OF CYTOPLASMIC MYOSIN:  
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Under the electron microscope it was clear (Ellis, 1970; and Burridge, 1971) that a general structure, with a head and tail region, is shared by both skeletal and smooth muscle myosin molecules (see Pollard, 1976).

The other line of evidence is that the presence of myosin in non-muscle cells comes from immunocytochemical studies. A wide variety of non-muscle cells show characteristic fluorescence patterns when treated with fluorescein-labeled myosin-specific antibodies (see Clark and Grosschedl-Stewart, 1974; Clark, 1976; Pollard and Pollard, 1976). That these immunofluorescence results support the biochemical findings concerning the presence of myosin in eukaryotic non-muscle cells.



## INTRODUCTION

As mentioned in the general introduction (Chapter 1), the occurrence of myosin in a wide variety of eukaryotic cell types is now well established (for reviews, see Pollard and Weihing, 1974; Clark and Spudich, 1977). The most convincing evidence to date comes from the biochemical isolation of myosin from these non-muscle cells. Purified non-muscle myosin exhibits ATPase activity which is stimulated by actin (Pollard and Weihing, 1974; Korn, 1978; Ostlund, Leung and Kipnis, 1978) and this myosin can physically bind to actin filaments (Pollard and Weihing, 1974). Under suitable ionic conditions this myosin will also form bi-polar thick filaments (Adelstein *et al.*, 1972; Clark and Spudich, 1977). Individual molecules of non-muscle myosin, which have been visualised under the electron microscope in some cases (Elliot, Offer and Burridge, 1976) show a general structure, with defined head and tail regions, which is similar to that of skeletal muscle myosin molecules (Lowey *et al.*, 1969).

The other line of evidence favouring the presence of myosin in non-muscle cells comes from immunocytochemical studies. A wide variety of non-muscle cells show characteristic fluorescence patterns when treated with fluorescein-labelled myosin-specific antibodies (Weber and Groeschel-Stewart, 1974; Sanger, 1975; Fujiwara and Pollard, 1976). Thus these immunofluorescence results support the biochemical findings concerning the presence of myosin in eukaryotic non-muscle cells. Immunofluorescence

studies are also valuable in demonstrating the general distribution of myosin inside these cells. In some cells, changes in the distribution of myosin occur during the cell cycle (Fujiwara and Pollard, 1976). However, in such studies, spatial resolution is, of necessity, limited to the light microscope level. Ultrastructural localization of myosin using ferritin-labelled, myosin-specific antibodies is only at a very early stage of development (Pollard *et al.*, 1976).

To support the view that myosin is involved in an actin/myosin-based system responsible for cell movements, it will be necessary to define the form taken by the non-muscle myosin and to plot its localization, at the ultrastructural level, throughout the cell. However, efforts directed towards the precise localization of myosin with the electron microscope have met with little success. Filaments resembling synthetic non-muscle myosin thick filaments have been observed intracellularly only in exceptional instances (Zucker-Franklin and Grusky, 1972; Rash *et al.*, 1972; Goldman, 1972). In most cells it has not been possible to identify bi-polar myosin thick filaments in sections of resin-embedded material (Pollard *et al.*, 1976). This is also the case in cells prepared for electron microscopy by either negative staining or critical-point drying. The assumption that myosin must occur in the form of thick filaments in non-muscle cells is based entirely on the finding that purified non-muscle myosin will form thick filaments in vitro under ionic conditions assumed to occur intracellularly. While

acknowledging the importance of in vitro model studies, it can be strongly argued that the ability of purified non-muscle myosin to form bipolar thick filaments under in vitro condition does not necessarily mean that myosin is present in this form within living cells. Precise intracellular conditions with respect to ionic composition, the concentration of myosin and the presence of regulatory factors, especially the likely occurrence of regulatory proteins, can be expected to determine the ultimate form of myosin inside cells. This does not preclude the possibility that in some non-muscle cells, myosin may be present as thick filaments, as was shown by Niederman and Pollard (1975) for platelets. However, the form which myosin takes in most non-muscle cells, including cultured chick embryo connective tissue cells, is still an open issue.

Thus, there is a need for a thorough investigation of the ultrastructure of non-muscle cells aimed at localizing myosin. In attempting to do this it was decided to make use of the carefully thought out approach applied by Hanson and Huxley (1955) for the localization of myosin in skeletal muscle cells. Making use of solutions containing pyrophosphate, these authors preferentially extracted myosin from glycerinated myofibrils and, with the electron microscope, compared the structure of the myosin-extracted fibril with that of the unextracted one. After myosin extraction, they found that the primary array of thick filaments in glycerinated myofibrils had entirely disappeared from the whole of the A band. Thus it was clearly demonstrated that myosin is present in the form of thick filaments in skeletal muscle cells. Here it is relevant to point out that in this laboratory it has been found that similar

solutions (i.e. those containing ATP or pyrophosphate) are effective in extracting the thick filaments from cultured cardiac muscle cells (Buckley, unpublished data).

In view of the above findings, an experimental plan similar to that of Hanson and Huxley (1955) was developed. This involved myosin extraction and ultrastructural comparison of myosin-extracted cells with either intact or glycerinated cells. The plan was applied to cultured chick embryo fibroblasts in the hope of establishing which cytoplasmic components of intact or glycerinated cells represent myosin. These ultrastructural studies on intact and extracted cells are supported by model studies involving electron microscopic observations of myosin thick filaments, monomers of myosin and mixtures of F-actin, and myosin monomers. One aim of these model studies was to find out whether it was possible to simulate structures resembling presumptive non-muscle myosin (as revealed by ultrastructural studies on intact cells) using purified myosin from skeletal-muscle. Then purified actin and myosin monomers were combined in a ratio similar to that present in non-muscle cells to see whether the resulting filamentous system, with attached myosin molecules, resembled that of cultured chick embryo connective tissue cells. As a biochemical supplement to this work, SDS polyacrylamide gel electrophoresis was carried out on extracts and extracted cells obtained after glycerination and myosin extraction to see whether or not myosin is extracted by the myosin extracting solutions.

4-11 no filaments. - These filaments are present both in bundles and in networks.



The present results are grouped as follows:-

Ultrastructural Studies of Cells

- (a) Intact (unextracted) cells;
- (b) Glycerinated cells;
- (c) Cells extracted by 'one-step' myosin extraction;
- (d) Cells extracted by 'two-step' myosin extraction.

Ultrastructural Studies of Motility Proteins

- (a) Synthetic thick filaments of myosin from rabbit skeletal muscle;
- (b) Monomers of myosin;
- (c) F-actin and myosin monomer mixtures.

SDS Polyacrylamide Gel Electrophoresis of Extracts and  
Extracted Cells obtained after Glycerination and Myosin  
Extraction

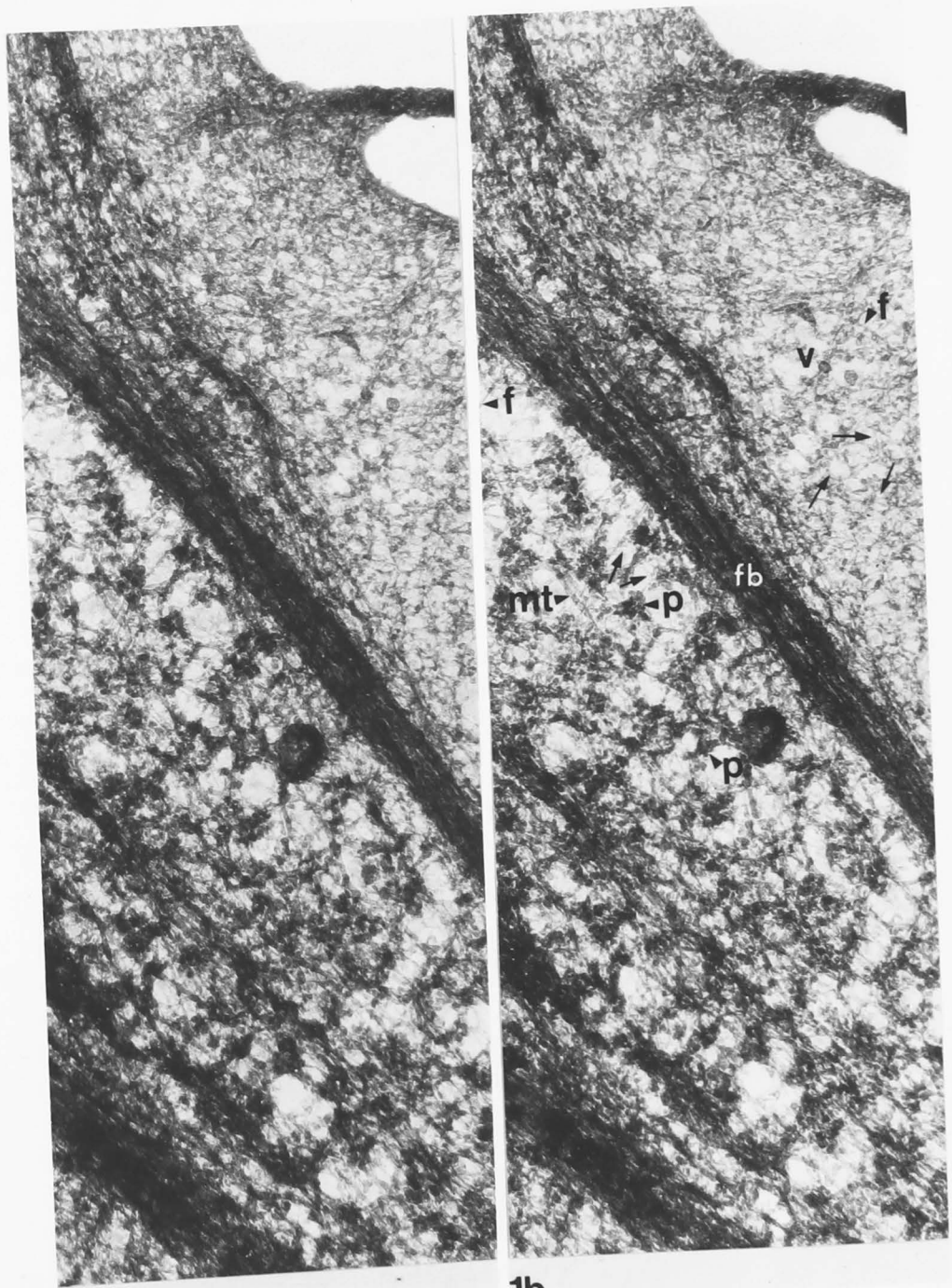
RESULTS

Ultrastructural Studies of Cells

(a) *Intact (unextracted) cells:* The ultrastructure of critical-point dried (CPD), unextracted, normal cultured chick embryo connective tissue cells has been described in detail previously (see Chapter 2). Here it is sufficient to recall the following features of the cell's filament systems, most of which are illustrated in the stereo pairs of micrographs (Figs. 1a and b):

- (i) The cytoplasm contains large numbers of 4-11 nm filaments. These filaments are present both in bundles and in networks.

Figs. 1a and b: Stereo pair of electron micrographs of a fixed, critical-point dried (CPD) cell showing the three dimensional arrangement of filaments in the cytoplasm. Within the veil-like extension of the sub-plasmalemmal cytoplasm (v), the filaments (f) are arranged in the form of a meshwork. They are heavily camouflaged and cross-linked by short cross-linking elements which appear as 'node-like' structures with fine tails, often arranged in 'dumb-bell' form (arrows). At the base of the veil, there is a filament bundle (fb). Deeper to the veil, within the sub-cortical cytoplasm is a three dimensional network of filaments (f). The 'node-like' structures are attached also to these sub-cortical filaments (arrows). Electron-dense clusters of polyribosomes (p), closely associated with the sub-cortical filaments and microtubules (mt) occur throughout the sub-cortical cytoplasm. X 55,500.



1a

1b

The bundles occur in the cortical region of the cell, both dorsally and ventrally. The networks have a three dimensional character and are distributed in both the sub-plasmalemmal and sub-cortical regions. Most of these filaments have been shown to contain F-actin (Buckley, Raju and Stewart, 1978; see also Chapters 4 and 5).

- (ii) No structures suggestive of bi-polar thick filaments are seen.
- (iii) Tiny 'node-like' structures are distributed throughout the cytoplasm, attached to the 4-11 nm filaments. These nodes are pleomorphic, but mostly ovoid or pear-shaped. The nodes have fine tails. The diameter of individual nodes is approximately 16 nm and that of their tails is from 1-2 nm. The nodes are sometimes joined to each other through their tails to form 'dumb-bell' shaped structures. These 'dumb-bells' cross-link the 4-11 nm filaments and also cross-link these filaments to microtubules and organelles such as mitochondria and polyribosomes.

(b) *Glycerinated cells*: Glycerination resulted in the formation of large holes in the plasma membrane of the cells. Despite this, the general cell shape was reasonably well preserved. Organelles had either disappeared or degenerated to form complex membrane-bound vesicles.



Since glycerination was carried out at 4°C for 24 hours, the microtubules were no longer visible. The overall features of the filament system in glycerinated cells are illustrated in Fig. 2 and the stereo pair of electron micrographs (Figs. 3a and b). Here the filament bundles and the sub-cortical network of filaments appear intact. The veils are not well preserved by glycerination. However, on occasions where veils can be observed, the sub-plasmalemmal network of filaments appears more or less normal. A remarkable feature is the 'roughness' of most of the filaments. This 'roughness' appears to be due to decoration of filaments with tiny 'node-like' structures. The shape of these is variable but they are mostly rounded, measuring ~15-16 nm in diameter. The association of these nodes with filaments appears more distinct in glycerinated cells than in intact cells. Some nodes show fine tails but, compared with intact cells, there are fewer 'dumb-bell' shaped structures cross linking adjacent filaments or organelles.

*(c) Cells extracted by 'one-step' myosin extraction:*

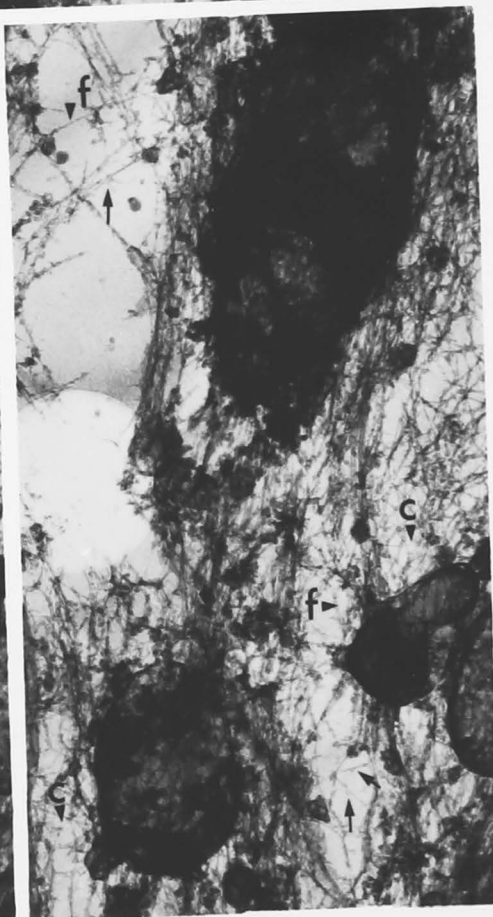
Within 2 hours of beginning extraction of the cells with the pyrophosphate-containing solution (for methods, see Chapter 2, p.32) a dramatic change occurred. The striking difference from the cells which were simply glycerinated is that the filaments were largely free of their associated 'node-like' structures. As a result, most of the filaments in the bundles and networks appeared smooth (Fig. 4). Cells extracted for 24 hours presented a similar picture but with more extensive removal of the nodal bodies. The resultant

Fig. 2: Electron micrograph of a cell which has been fixed and CPD after glycerination in the presence of standard salt solution for 24 hours (for methods, see Chapter 2, p.30). Glycerination affects the cell membrane and organelles present within the cell. Large holes are present in the plasma membrane and the organelles are transformed to membrane-bound vesicles (mbv). However, the network of filaments (f), remains little affected by the procedure of glycerination. X 44,100.

Figs. 3a and b: Stereo pair of electron micrographs of a part of the cell depicted in Fig. 2. The striking feature is the decoration of many of the filaments (f) of the three dimensional filamentous network by 'node-like' structures. These structures, by tail-to-tail association form 'dumb-bell' shaped structures, which cross-link (c) adjacent filaments. X 44,100.



3a

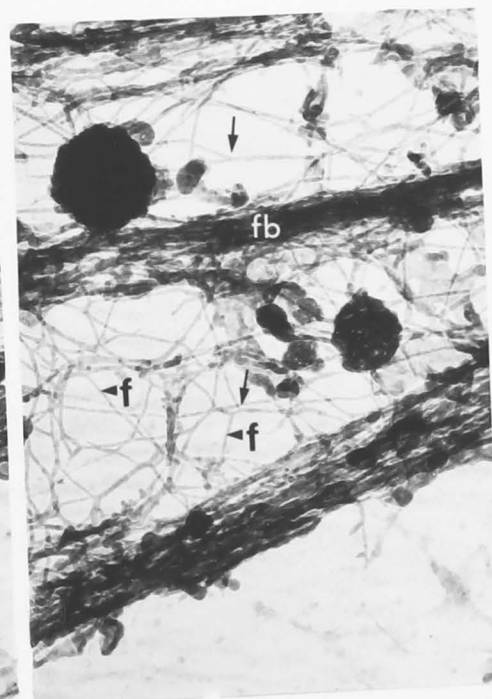
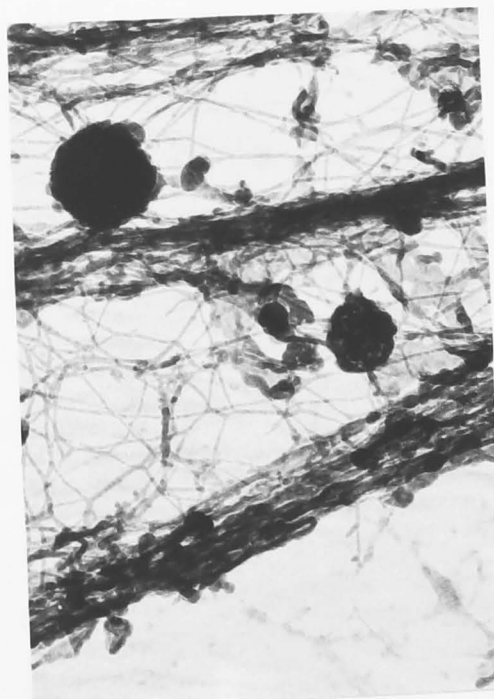
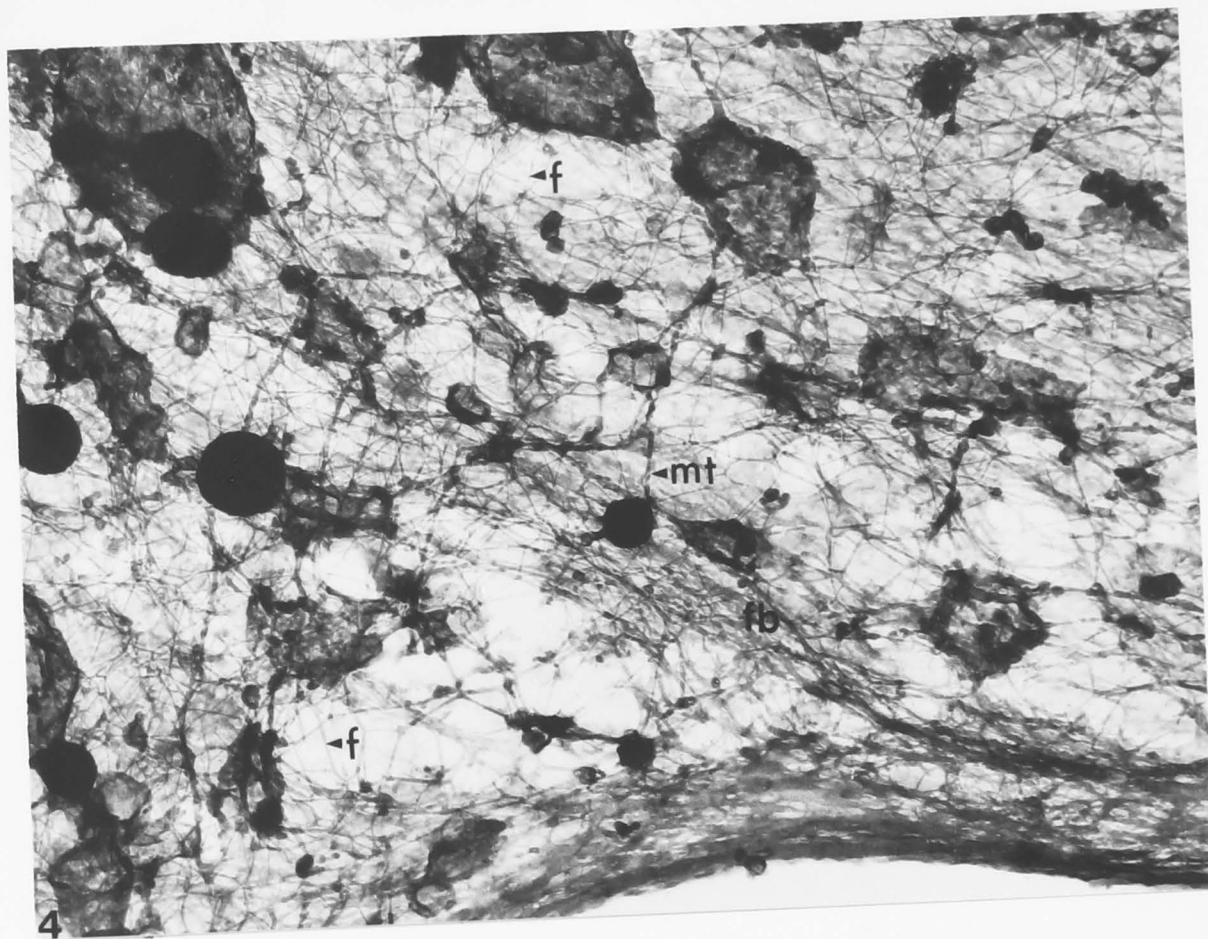


3b

Fig. 4: Portion of a CPD cell which was subjected to 'one-step' myosin extraction for 2 hours at 4°C (for methods, see Chapter 2, p.32). The filaments (f) are totally free of 'node-like' structures and hence they appear smooth. Disintegrating microtubules (mt) are also seen. The filaments ranging from 4-11 nm in diameter form a three dimensional network by branching and anastomosing with one another. A loose filament bundle (fb) is also visible. X 44,100.

Figs. 5a and b: Stereo pair of electron micrographs of a portion of a cell which has been treated in the same way as that shown in Fig. 4 except that myosin extraction was carried out for a longer duration (24 hours). The filaments (f) are completely free of 'node-like' structures. Branching of filaments (arrows) is clearly seen. The filaments are arranged in the form of a bundle (fb) and a three dimensional network. X 54,600.





filaments within the three dimensional network appeared extremely smooth (Figs. 5a and b). The branching and anastomosing of filaments are clearly evident.

*(d) Cells extracted by 'two-step' myosin extraction:*

It was decided to glycerinate the cells first and then extract them for myosin, as done by Hanson and Huxley (1955) with skeletal muscle myofibrils, to find out whether this type of extraction gave results different from the 'one-step' procedure. When the results were compared, it was found that the 'two-step' and 'one-step' myosin extraction procedures yielded closely similar results.

As the stereo pair of electron micrographs (Figs. 6a and b) indicate, the filament bundles and the network of filaments are well preserved in the cells extracted by 'two-step' myosin extraction. At this magnification, it is not possible to evaluate whether or not the filaments are smooth, i.e. free of any attached 'node-like' structures. However, at higher magnification (Figs. 7a and b), it is clearly shown that the filaments are smooth and completely free of nodes. The cross-linking 'dumb-bell' shaped elements are also absent. A comparison of these figures with those of Figs. 2, 3a and b demonstrates beyond any doubt that 'node-like' structures which are associated with the filaments in glycerinated cells are almost completely absent in cells which have been glycerinated and myosin extracted.

Figs. 6a and b: Low power stereo electron micrographs of a cell which has been CPD after 'two-step' myosin extraction for 24 hours at 4°C (for methods, see Chapter 2, p.32). The cortical filament bundles (fb) and the network of filaments (f), can be clearly seen. Although at this magnification it is not easy to be certain that the filaments are completely free of 'node-like' structures, the general impression is that most filaments in both the bundles and the network are smooth. X 35,000.

Figs. 7a and b: Stereo pair of electron micrographs taken under high power showing a portion of a cell which has been fixed and CPD after 'two-step' myosin extraction at 4°C for 24 hours. The filaments (f) are almost completely free of attached nodes (cf. Figs. 3a and b). The residual smooth filaments are clearly seen as a branching (arrows) and anastomosing network. X 70,000.

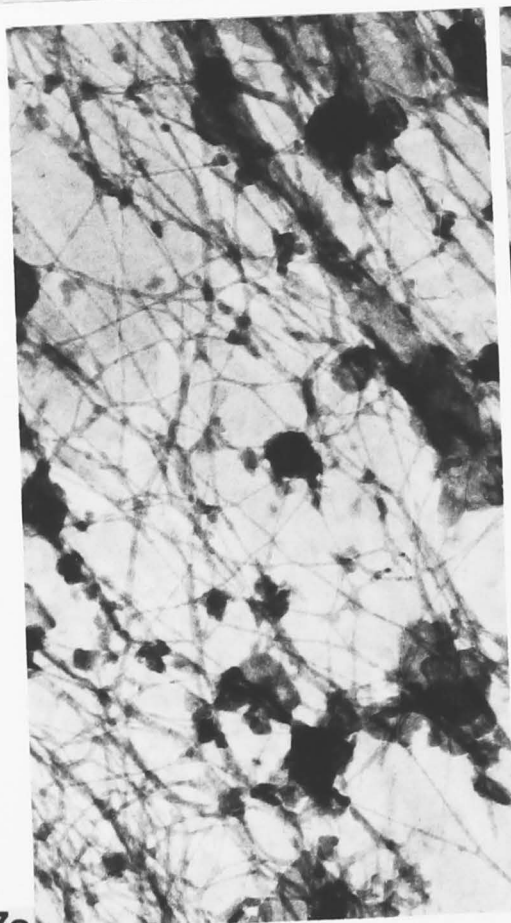




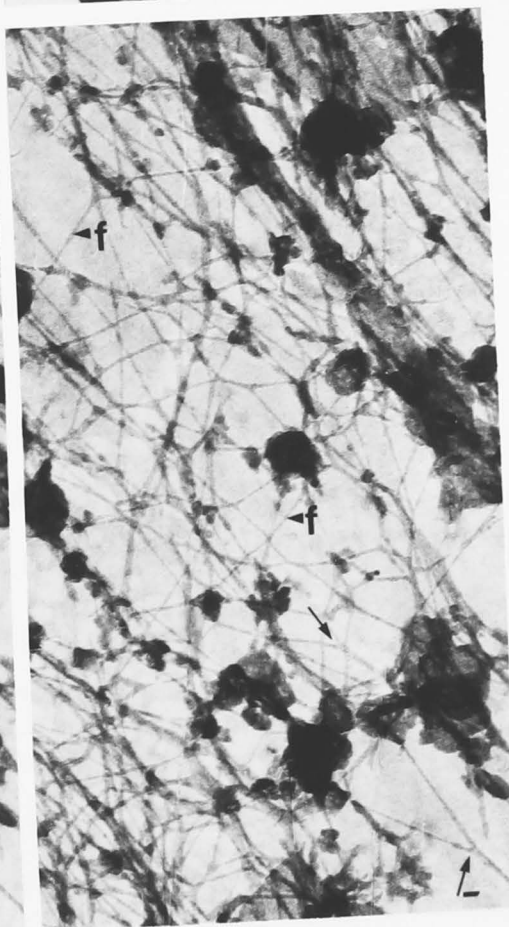
6a



6b



7a



7b



## Ultrastructural Studies of Motility Proteins

### *(a) Synthetic thick filaments of myosin from rabbit*

*skeletal muscle:* The ultrastructure of synthetic myosin thick filaments derived from skeletal muscle was studied after (i) negative staining and (ii) critical-point drying. Critical-point drying was done to determine whether this procedure would preserve myosin thick filaments in a recognizable form. The negative staining was carried out for comparative purposes, since it is well known that negative staining preserves myosin thick filaments very efficiently (Huxley, 1963).

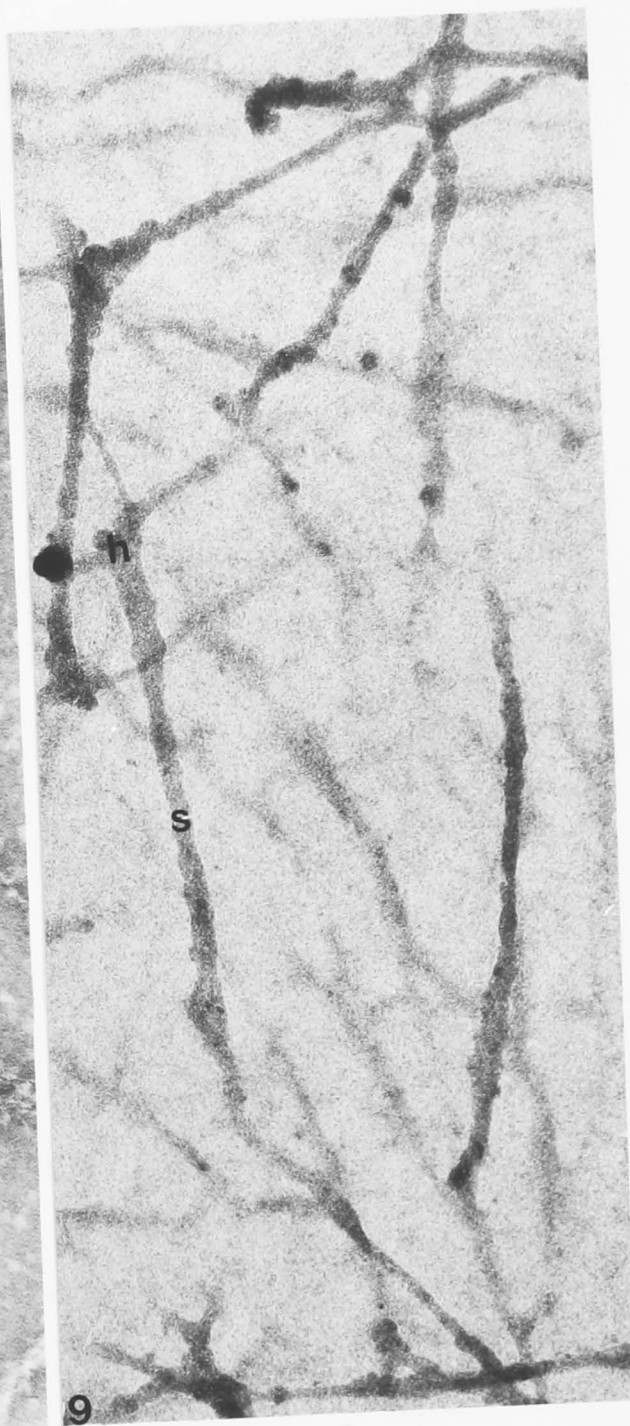
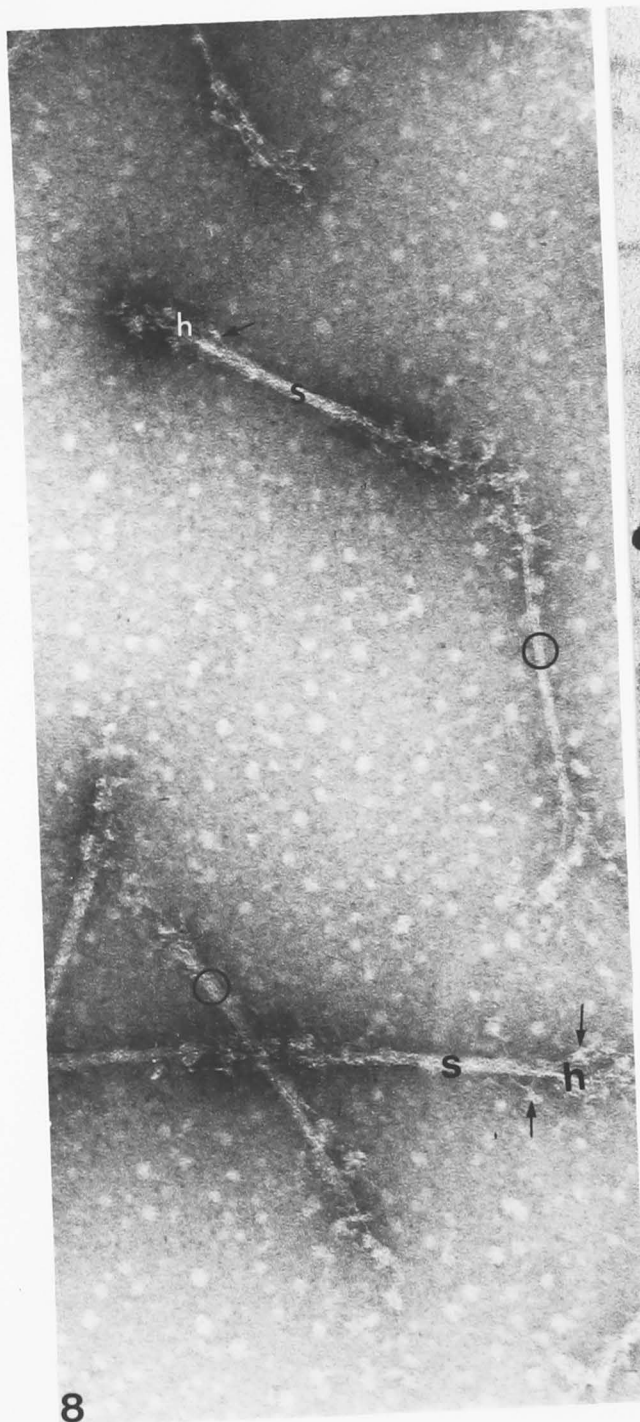
#### (i) Negatively stained myosin thick filaments:

The myosin thick filaments appear as well-defined bi-polar structures with bulbous ends separated by a straight shaft region (Fig. 8). The mean diameter of the shaft is  $13.62 \pm 0.15$  nm (50 filaments were measured). The shaft is relatively bare except for the projecting heads of occasional individual myosin molecules. The heads of the myosin molecules are also distinctly seen in the bulbous end regions.

#### (ii) CPD myosin thick filaments: Although the details of the structure of these myosin thick filaments are not well preserved, their overall shape is reasonably well maintained (Fig. 9). Thus one can still recognize their bi-polar form. An important detail which is lost is that the heads of individual myosin molecules, which

Fig. 8: Electron micrograph showing negatively stained bipolar thick filaments of purified skeletal muscle myosin (prepared from rabbit skeletal muscle as described in Chapter 2, p.43) showing head (h) and shaft (s) regions. Subfilaments (circled areas) can be appreciated within the shaft region. Individual myosin molecules (arrows) projecting out from the head region can also be seen. X 154,000.

Fig. 9: Electron micrograph showing fixed CPD myosin thick filaments consisting of head (h) and shaft (s) regions. Other details are not clearly seen when compared with negatively stained preparations (cf. Fig. 8). X 154,000.



clearly project out from the shaft in negatively stained preparations, seem to be merged into the shaft in these CPD preparations. Apart from such differences, the shaft diameter of both negatively stained and CPD thick filaments varies little. When the shafts of 50 CPD filaments were measured, the mean diameter was found to be  $13.11 \pm 0.17$  nm, that is, only about 0.5 nm less than that of negatively stained preparations.

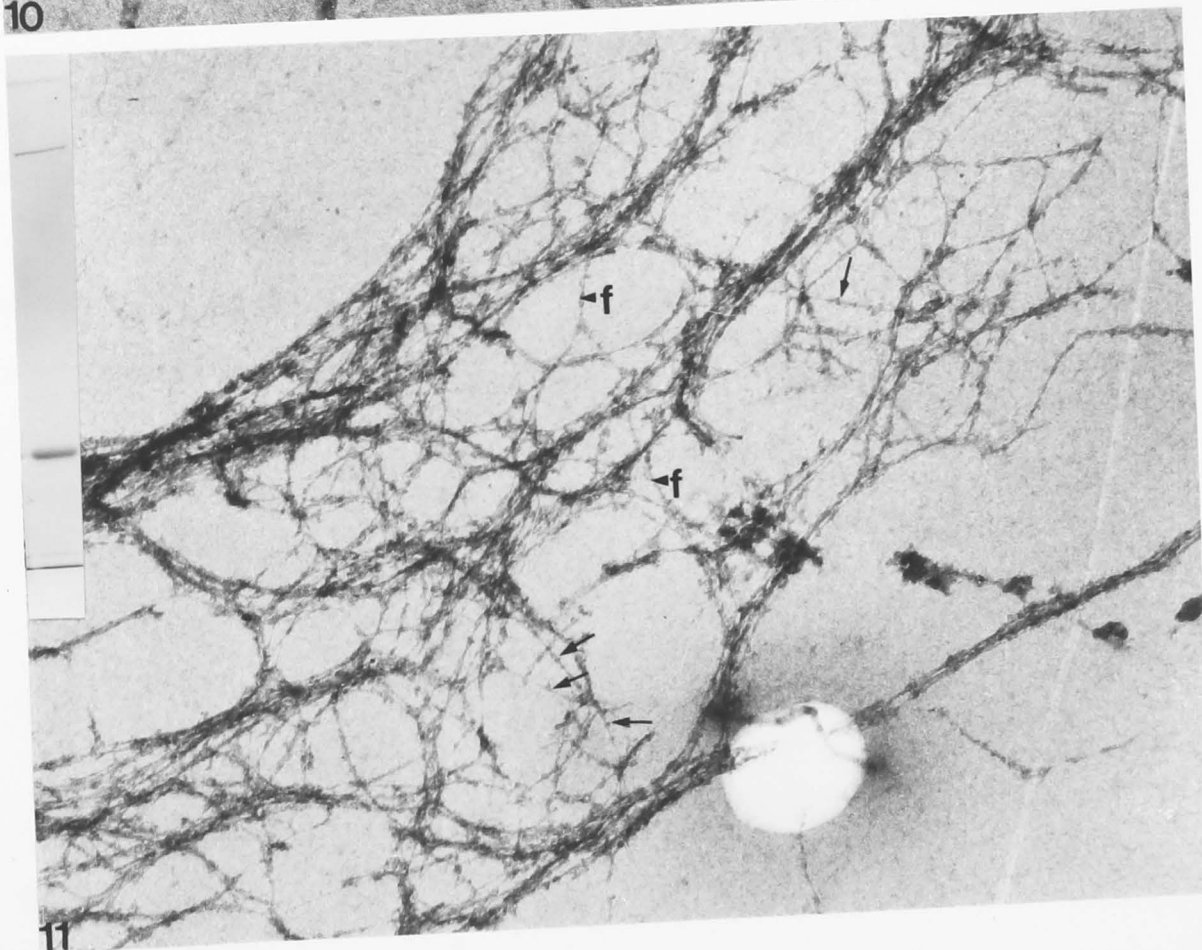
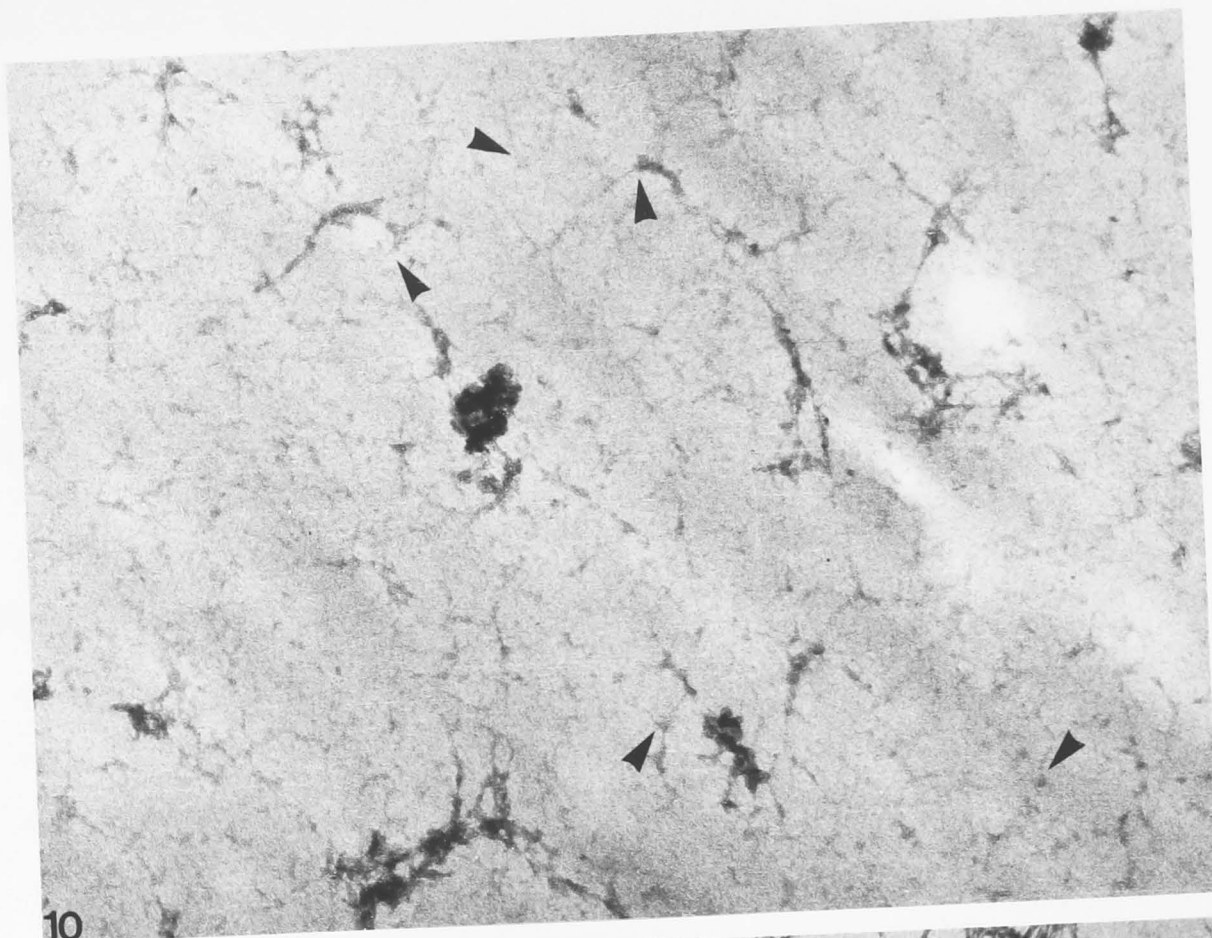
These observations indicate that even in CPD cells it should be possible to visualize myosin thick filaments if they are present.

(b) *Monomers of myosin:* Myosin monomers isolated and purified from rabbit skeletal muscle were fixed and CPD to determine their appearance with a view to considering whether similar structures are present in the fixed, CPD chick embryo connective tissue cell preparations. It must be pointed out that there are considerable difficulties associated with visualizing myosin monomers with the electron microscope unless a shadow casting technique is used (Slayter and Lowey, 1967). Nevertheless, careful observation of Fig. 10, reveals the structure of myosin molecules with characteristic head and tail regions. It is hard to differentiate between the two heads of a single molecule and in most instances the two heads appear to be fused together. The diameter of the fused heads averages about 16 nm and the diameter of the tail varies from 1-1.5 nm.



Fig. 10: Electron micrograph of fixed and CPD monomers of skeletal muscle myosin showing structures (arrowheads), which are highly suggestive of possessing heads and tails. In the monomers, the two heads of individual myosin molecules appear fused together, these appearing 'node-like' with an attached fine tail.  
X 54,600.

Fig. 11: CPD mixture of F-actin and myosin monomer (for methods, see Chapter 3, p.43) showing heads of myosin molecules (arrows) attached to F-actin filaments (f), which are arranged in the form of a network. This structure resembles the network of filaments with attached 'node-like' structures in glycerinated cells (cf. Figs. 3a and b). The decoration seems to be regular in some areas but not so in others. The purity of the proteins used in this model was checked with SDS polyacrylamide (10%) gel electrophoresis (for methods, see Chapter 2, p.54) and this is shown in the inset. (Electron micrograph.  
X 54,600.)



'Dumb-bell' shaped dimer-like associations are also present. In general, the form and dimensions of these structures appear similar to the 'node-like' structures attached to filaments in both intact and glycerinated cells.

(c) *F-actin and myosin monomer mixtures:* Purified skeletal muscle myosin monomers and F-actin were mixed at a molar ratio of 1:50 which is approximately the same as in many non-muscle cells. (The molar ratio of total actin: myosin in most non-muscle cells is 100:1 (Pollard, 1976b); of the total actin only 50% is in a polymerized form (Bray and Thomas, 1976)). The mixture was fixed and CPD (for details of preparation, see Chapter 2, p.52). As with purified actin allowed to polymerize by itself, a three dimensional network of filaments was formed (Fig. 11). In addition, within the three dimensional network there are many tiny nodal bodies, presumably individual myosin molecules, closely attached to the actin filaments. These are regularly arranged in some instances, but not so in others. This network bears a close resemblance to the network of filaments with attached 'node-like' structures which is observed in CPD, glycerinated cells. As mentioned above, the dimensions of the nodes found in cells are similar to those of the myosin molecules decorating these actin filaments.

#### SDS Polyacrylamide Gel Electrophoresis of Extracts and Extracted Cells obtained after Glycerination and Myosin Extraction

A comparison of the extract obtained after glycerination

on the one hand and the extract obtained after glycerol/myosin extraction on the other, reveals that a band comigrating with skeletal muscle myosin is present only in the extract obtained by myosin extraction but not in the extract after glycerol extraction (Fig. 12). However, the myosin extracted cells (residue after extraction) still shows the presence of small quantities of a band comigrating with skeletal muscle myosin.

The major band which is present in the cells as well as the extract samples comigrates with bovine serum albumin. This may be due to the high concentration (10%) of foetal calf serum present in the culture medium. Since glycerol makes the cells permeable, many soluble proteins, covering a wide range of molecular weights, are also present in both extracts. Actin, which is known to occur in significant quantities in the soluble, or G-form, in non-muscle cells (Bray and Thomas, 1976), is therefore present in extracts obtained with glycerol as well as with the myosin extracting solution.

The extracts were also analysed on a 5% gel to see the various high molecular weight proteins which were extracted. As Fig. 13 indicates, most of the high molecular weight proteins other than myosin are found in extracts obtained with glycerol as well as with the myosin extracting solution. Myosin is the only additional band in the extract obtained with the myosin extracting solution.



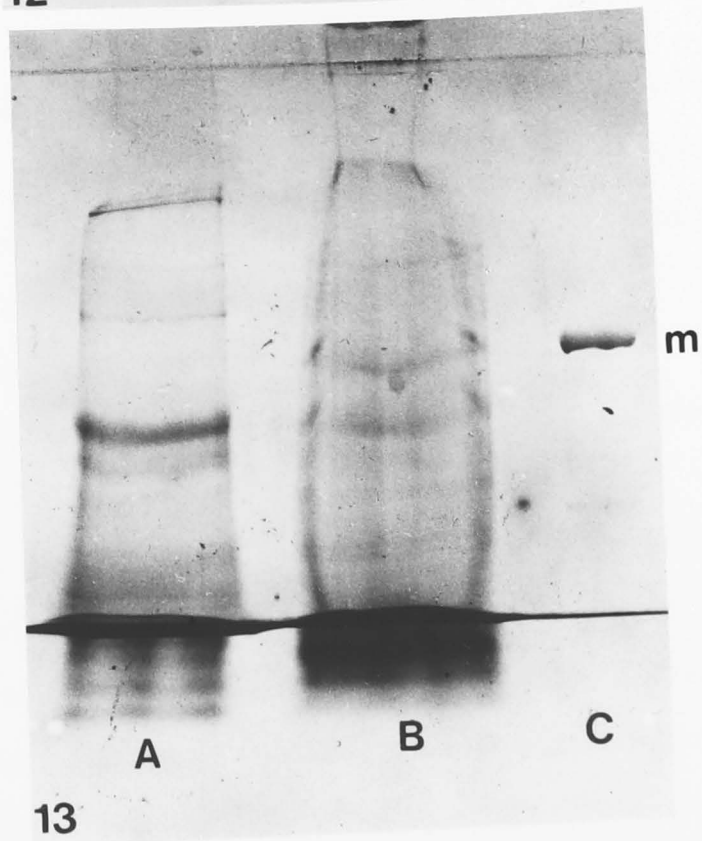
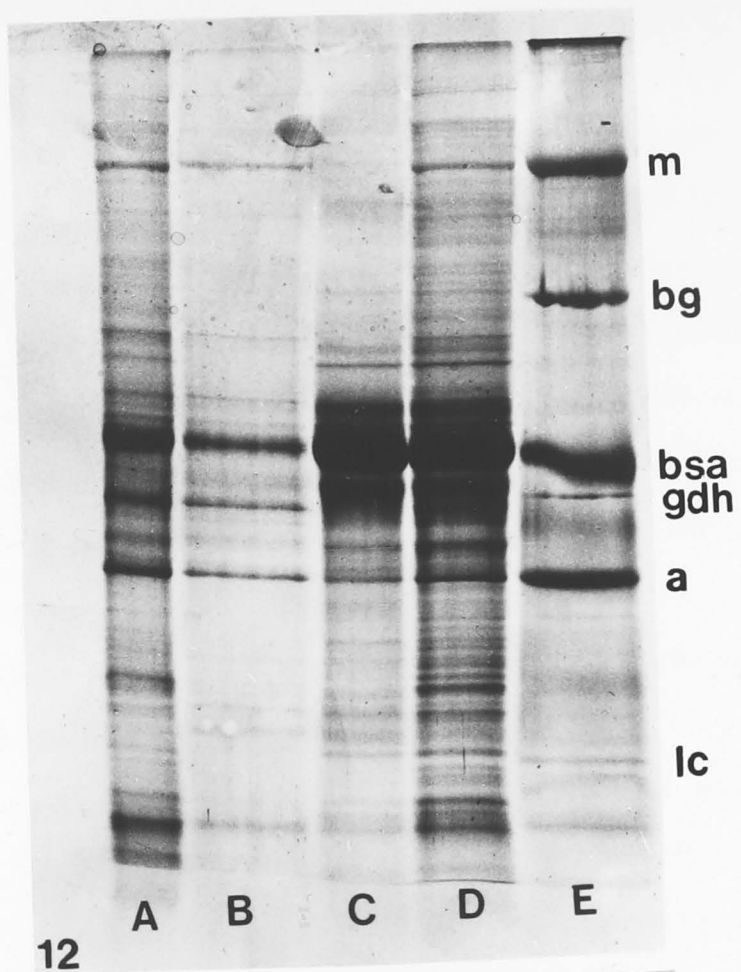
Fig. 12: Profile of SDS polyacrylamide gradient (6½-15%) gel electrophoresis (Method described in Chapter 2, p.54) showing:

- A. Cells (residue) extracted by glycerol in standard salt solution (glycerol/SSS).
- B. Cells (residue) extracted by myosin extracting solution.
- C. Extract of cells obtained after glycerol/SSS extraction.
- D. Extract of cells obtained after treating the cells with myosin extracting solution.
- E. Standards consisting of purified skeletal muscle myosin heavy chain (m) (200K),  $\beta$ -galactosidase (bg) (130K), bovine serum albumin (bsa) (68K), glutamate dehydrogenase (gdh) (53K), actin (a) (42K) and myosin light chain (lc) (21K).

The presence of a significant quantity of myosin can be seen in the myosin extract (D) but not in the glycerol extract (C). Myosin-extracted cells (B), still show a small quantity of myosin. The major band in all preparations (extracts and extracted cells) is serum albumin, which must have had its origin from culture medium, since this medium contains 10% foetal calf serum.

Fig. 13: SDS polyacrylamide (5%) gel electrophoresis of extracts obtained after extraction of cells with glycerol/SSS and myosin extracting solution.

- A. Cell extract obtained after extracting with glycerol/SSS showing no band comigrating with myosin. However, as in B, it shows a few bands at the high molecule weight regions.
- B. Cell extract obtained after extracting with myosin extracting solution showing a band comigrating with the myosin standard. A few other bands are also present at the high molecular weight region.
- C. Purified skeletal muscle myosin (m) (200K) as standard.



## DISCUSSION

The major conclusion derived from the present work is that myosin in cultured chick embryo connective tissue cell is present in some highly dispersed form, possibly as monomers or dimers. The evidence to support this view is:

- (1) Although the results of SDS polyacrylamide gel electrophoresis indicate that cultured chick embryo connective tissue cells contain myosin (Ostlund, Pastan and Adelstein, 1974; see also Chapter 3), no thick filaments can be seen when the cells are examined by electron microscopy.
- (2) The cells contain 'node-like' structures with fine tails and 'dumb-bell' shaped structures which are readily extracted with pyrophosphate-based myosin extracting solutions.
- (3) The loss of nodes following treatment with myosin-extracting solution is paralleled by the appearance of a band comigrating with muscle myosin heavy chain band in SDS polyacrylamide gels.
- (4) There is a general resemblance between 'node-like' structures in CPD cells and CPD monomers of purified skeletal muscle myosin.
- (5) When skeletal muscle myosin monomers and F-actin are mixed together (in a molar ratio approximately equivalent to that which occurs in non-muscle cells), fixed and CPD, the resultant structure resembles the filamentous system in intact and glycerinated cells.

Determining the form taken by myosin in non-muscle

cells has always been difficult because thick filaments resembling skeletal muscle myosin filaments have rarely been observed in these cells (Pollard *et al.*, 1976). In the few cases where myosin filaments have been sighted, such as in osmotically shocked platelets (Zucker-Franklin and Grusky, 1972), cytochalasin B-treated cultured cells (Goldman, 1972), isolated intestinal brush borders (Mooseker and Tilney, 1975) and endothelial cells and pericytes of rat brain capillaries and postcapillary venules (Le Beaux and Willemot, 1977), the cells have been pre-treated in various non-physiological ways prior to fixation. It seems that whenever tissues or cells have been fixed without such pre-treatment myosin thick filaments have been absent.

This failure to observe myosin thick filaments in non-muscle cells with the electron microscope is unlikely to be due to the fixation and dehydration procedures involved in the processing of cells for electron microscopy. Pollard (1974) demonstrated that purified platelet myosin survives glutaraldehyde and osmium fixation. It has also been shown in the experiments described above (see Fig. 9) that fixation, acetone dehydration and CPD do not drastically alter the ultrastructure of skeletal muscle myosin thick filaments. Accordingly, if myosin thick filaments are present in the living cell, they should also be visible in fixed and CPD cells. However, no thick filaments were observed in fixed and CPD chick embryo connective tissue cells (Figs. 1a and b).

With the above considerations in mind, it seems highly probable that myosin in cultured chick embryo fibroblasts



is present not as thick filaments but in some more dispersed form, possibly as monomers or dimers. This possibility has been considered previously for different types of non-muscle cells (Nachmias, 1972; Small, Isenberg and Celis, 1978 and Korn, 1978). Nachmias (1972) showed that Physarum myosin did not form thick filaments, even with concentrations of myosin of 0.5 mg/ml and under conditions designed to enhance ionic or hydrogen bonding between myosin molecules. Small, Isenberg and Celis (1978) failed to observe any myosin thick filaments at the leading edges of 3T3 cells and human skin fibroblasts and hence proposed that myosin may occur as monomers in these cells.

If myosin is present as monomers or dimers in cultured chick embryo connective tissue cells, the question to be asked is whether the nodes and 'dumb-bell' shaped structures described in the results of this chapter represent myosin molecules. When the cells were extracted for myosin, the above-mentioned structures disappeared from the cells and this was paralleled by the appearance of bands comigrating with myosin heavy chain bands in the extract on SDS polyacrylamide gel electrophoresis. This is highly suggestive that 'node-like' and 'dumb-bell'-like structures do represent monomers and dimers of myosin in these culture cells.

When the extracts after glycerination and myosin extraction were analysed by SDS polyacrylamide gel electrophoresis, many protein bands covering a wide range of molecular weights were seen (Fig. 12). It is assumed that most of these bands are due to soluble proteins which diffused out when the cells were rendered permeable by

glycerol. The most significant difference between the glycerol and myosin extraction is the presence of bands at the myosin heavy chain region in the myosin extract and their absence in the glycerol extract. The amount of myosin extracted was not estimated quantitatively in relation to the total myosin content of the cells. Hence, it is not possible to establish whether the presence of a band in the myosin heavy chain region (200,000 daltons) remaining in the myosin-extracted cells is due to incomplete extraction of myosin. Alternatively, two other factors might be involved. First, the cells may contain other proteins having a similar sub-unit molecular weight to that of the myosin heavy chain. Secondly, myosin molecules, once extracted, may become caught up in the interstices of the extracted cells (Buckley and Raju, 1976). Some of the extracted myosin molecules, even after coming out of the cells may have become stuck to the surface of the culture bottles, rendered hydrophilic by culture medium. These extracted myosin molecules would then be solubilized when the myosin-extracted cells are dissolved in the SDS.

Since high molecular weight proteins other than myosin are extracted, it is possible that some of the 'node-like' structures, all of which were previously interpreted as myosin molecules (Buckley and Raju, 1976), could include one or more of the following high molecular weight actin binding proteins:

- (1) A 280,000 dalton actin-binding protein described by Stossel and Hartwig (1975).

- (2)  $\alpha$ -actinin, which has a molecular weight of  $\sim 100,000$  daltons, and
- (3) other high molecular weight proteins yet to be clearly defined (Korn, 1978).

Although there is a possibility that the 'node-like' structures may include such high molecular weight proteins, it can be argued that most of the 'node-like' structures represent myosin for the following reasons:

- (i) As illustrated in the gel, other than myosin itself, most of the high molecular weight proteins extracted by the myosin-extracting solution are also extracted by the glycerol-standard salt solution (Fig. 13). This finding, correlated with the ultrastructural observation that extraction with the glycerol-standard salt solution does not remove the 'node-like' structures from the cells (these structures being extracted only by myosin-extracting solutions) suggests that it is highly likely that most of the 'node-like' structures represent myosin rather than high molecular weight actin-binding proteins.

- (ii) There is a broad resemblance between the 'node-like' structures and the myosin monomers isolated from the

skeletal muscle cells. (See results of this chapter)

- (iii) When muscle actin filaments are incubated with muscle myosin monomers in a ratio similar to that believed to occur in non-muscle cells (Pollard, 1976b; Bray and Thomas, 1976) a structure consisting of a network of filaments with attached nodes, similar to the filamentous structures found in intact and glycerinated chick embryo connective tissue cells is formed.
- (iv) Whereas most of the 'node-like' structures in intact and glycerinated cells show distinct tails, the high molecular weight actin-binding proteins have not been found to possess any tails (Stossel and Hartwig, 1975).

It is acknowledged, however, that unless it is shown by some specific myosin-labelling technique at the electron microscope level (e.g. using ferritin labelled myosin antibodies) it cannot be concluded firmly that these 'node-like' structures represent myosin.

Models used here for the purpose of assessing the form which might be taken by myosin in cultured chick embryo connective tissue cells involved using monomers of purified skeletal muscle myosin. Although the use of skeletal muscle myosin rather than a non-muscle myosin as a model may be questioned, it seemed justifiable to use a skeletal



muscle myosin model. This is because it has been clearly shown that except for the differences in the length of the tails, the morphology of muscle myosin (Lowey *et al.*, 1969), vertebrate non-muscle myosin from various tissues (Elliot, Offer and Burridge, 1976), and Acanthamoeba myosin - II (Pollard, Stafford and Porter, 1978) are closely similar. At the outset of this project attempts were made to purify myosin in sufficient quantities from cultured chick embryo connective tissue cells. However, these were not successful because the quantity of starting material was limited, even when mass culturing techniques were used.

In conclusion, it must be pointed out that actomyosin based contraction does not necessarily require a structure as elaborate as a myosin thick filament. Two actin filaments may be drawn together by a tail to tail dimer of myosin, as long as the actin filaments have appropriate (opposite) polarities (Nachmias, 1972). There is also a possibility that several myosin molecules could be attached to one actin filament by the rod segments of their heavy chains, perhaps through auxilliary proteins, thereby leaving the myosin heads free to interact with another actin filament. Angular displacement of myosin heads would result in the movement of the second actin filament in relation to the first one. This possibility has been proposed by Korn (1978). Thus, the occurrence of myosin as monomers, dimers or small oligomers in cultured chick embryo connective tissue cells is conceivable and could be functionally meaningful.

Future work in the following areas would further substantiate the proposed form and distribution of myosin in cultured connective tissue cells:

(1) Development of a suitable electron microscope probe for myosin which can be (i) either a ferritin-labelled myosin-specific antibody, as mentioned earlier or (ii) a heavy metal thiol label which can give specific electron-dense labelling of the heads of myosin molecules.

(2) Isolation of myosin from non-muscle cells in sufficient quantities to study:

(i) the form taken by purified myosin at different concentrations to determine the minimum concentration for filament formation under various ionic conditions, and

(ii) the behaviour of myosin molecules in the presence of a large excess of F-actin and, under conditions favouring self aggregation of myosin molecules, to determine whether the binding affinity of myosin molecules to F-actin is greater than the tendency to self aggregate to form myosin thick filaments (cf. Matsumura and Hatano, 1978)

(3) Ultrastructural investigation of CPD purified high molecular weight actin-binding proteins such as  $\alpha$ -actinin and the protein described by Stossel and Hartwig (1975) to assess the possibility that some of the 'node-like' structures represent these proteins .

The electron microscopy and phase contrast studies described in Chapters 4 and 5 led to the conclusion that part of the intermediate filaments in cultured chick embryo fibroblasts contain a core of protein associated with specific accessory proteins. Accordingly, it was decided to see whether intermediate filaments in other types of cells also contain protein. Chicken gizzard was considered to be a good source because of the abundance of characteristic filaments in the smooth muscle cells of this organ (Shenoy and Mathew, 1974). It was believed

CHAPTER 7 - THE INTERMEDIATE FILAMENTS OF CHICKEN  
GIZZARD SMOOTH MUSCLE:  
A PRELIMINARY ULTRASTRUCTURAL AND  
BIOCHEMICAL ANALYSIS

Biochemical characterization of intermediate filaments of smooth muscle was first carried out by Cooke and Chase (1971). They demonstrated their insolubility in solutions of high ionic strength. Rice and Todd (1971) observed two high molecular weight bands, corresponding to about 55,000 and 105,000 daltons (35 and 100) in the isolated intermediate filaments of chicken gizzard. Later, Chase (1975) reported that a protein having a sub-unit molecular weight of 55k is the major constituent of the intermediate filaments of these cells. At about the same time, however, Lazarides and Hubbard (1974) came to the conclusion that the molecular weight of the major sub-unit of these

## INTRODUCTION

The HMM-labelling and DNAase I studies described in Chapters 4 and 5 led to the conclusion that most of the intermediate filaments in cultured chick embryo fibroblasts contain a core of F-actin associated with specific accessory protein(s). Accordingly, it was decided to see whether intermediate filaments in other types of cells also contain F-actin. Chicken gizzard was considered to be a good source because of the abundance of intermediate filaments in the smooth muscle cells of this organ (Shoenberg and Needham, 1976). It was believed to be relatively easy to isolate a homogenous population of intermediate filaments from these cells (Cooke and Chase, 1971; Rice and Brady, 1973; Cooke, 1976; Small and Sobieszek, 1977).

Biochemical characterization of intermediate filaments of smooth muscle was first carried out by Cooke and Chase (1971), who demonstrated their insolubility in solutions of high ionic strength. Rice and Brady (1973) observed two high molecular weight bands, corresponding to about 85,000 and 105,000 daltons (85 and 100K) in the isolated intermediate filaments of chicken gizzard. Later, Cooke (1976) reported that a protein having a sub-unit molecular weight of 55K is the major constituent of the intermediate filaments of these cells. At about the same time, however, Lazarides and Hubbard (1976) came to the conclusion that the molecular weight of the major sub-unit of these



intermediate filaments is 50K; they called this protein 'desmin'. Schollmeyer, *et al.* (1976) favoured the view that a 55K protein is the major sub-unit of chicken gizzard intermediate filaments and that, in addition, proteins having sub-unit molecular weights of 100K and 200K are also present. According to Small and Sobieszek (1977), a 55K protein is the sole constituent of intermediate filaments because it was claimed to be possible to resynthesize such filaments using 55K protein alone; these authors named their protein 'skeletin'. These studies suggest that either the 50K or the 55K (the difference may be due to differences in the electrophoretic methods) sub-units provide the major protein in the intermediate filaments of chicken gizzard. However, both Cooke (1976) and Lazarides and Hubbard (1976) found a significant quantity of actin (detected by SDS polyacrylamide gel electrophoresis) which copurified along with the 55K (or 50K) protein of intermediate filaments, even after preliminary extensive actomyosin extraction. While Cooke (1976) treated the presence of actin as a contaminant, Lazarides and Hubbard (1976) did not preclude the possibility of actin being a constituent of the chicken gizzard intermediate filaments. Therefore, the question of whether actin forms an integral component of these intermediate filaments is an undecided one. Accordingly, to see whether actin might occur within these filaments in the form of an F-actin core (as was shown in the case of intermediate filaments of chick embryo connective tissue cells (see Chapters 4 and 5)), the gizzard smooth muscle intermediate filaments were

subjected to DNAase I treatment and HMM labelling following gentle trypsinization. These procedures were adapted from the techniques used to demonstrate the presence of F-actin in the intermediate filaments of chick embryo connective tissue cells (see Chapters 4 and 5).

The results of this work are presented under the following headings:

- (a) Isolation of intermediate filaments;
- (b) Ultrastructure of intermediate filaments;
- (c) Effect of DNAase I on intermediate filaments;
- (d) Effect of trypsin and HMM on intermediate filaments;
- (e) Effect of aqueous extraction on the structure of intermediate filaments.

## RESULTS

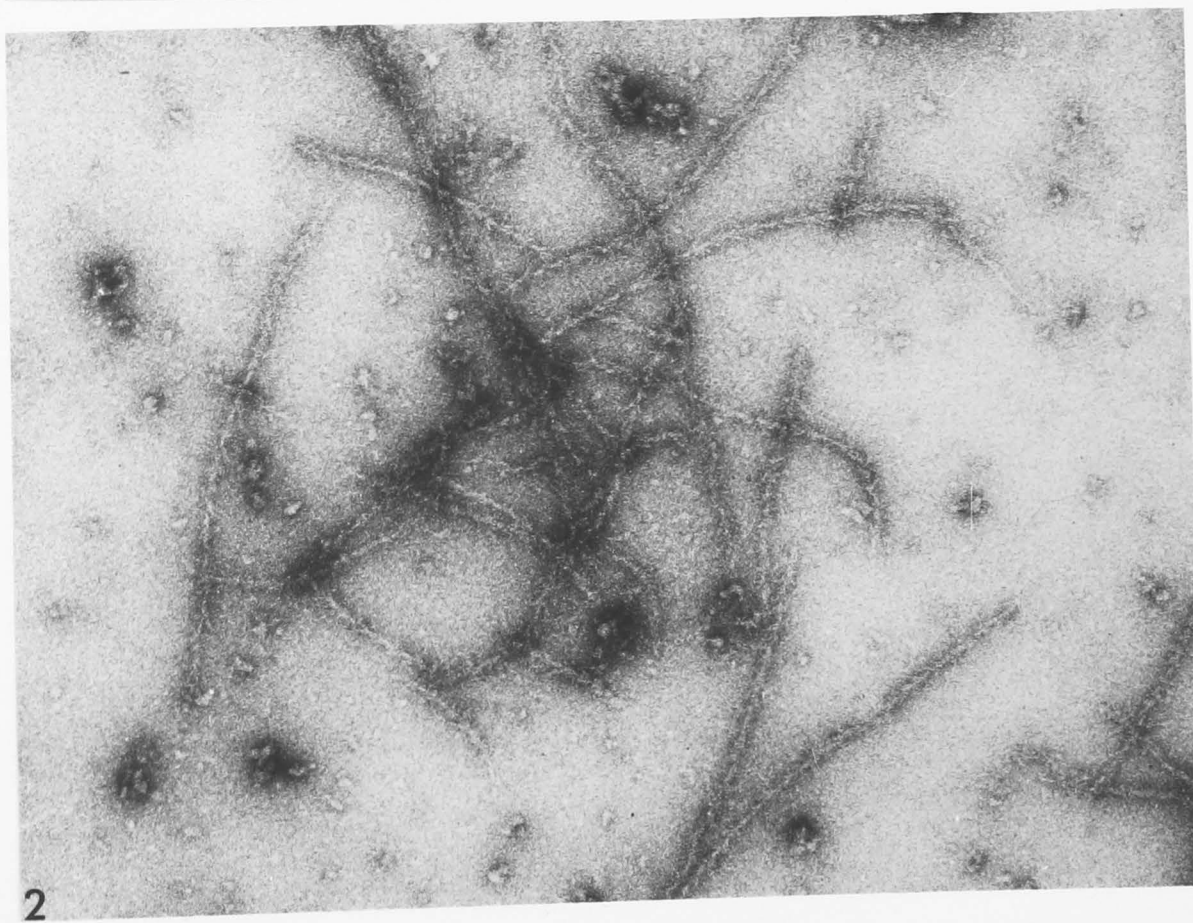
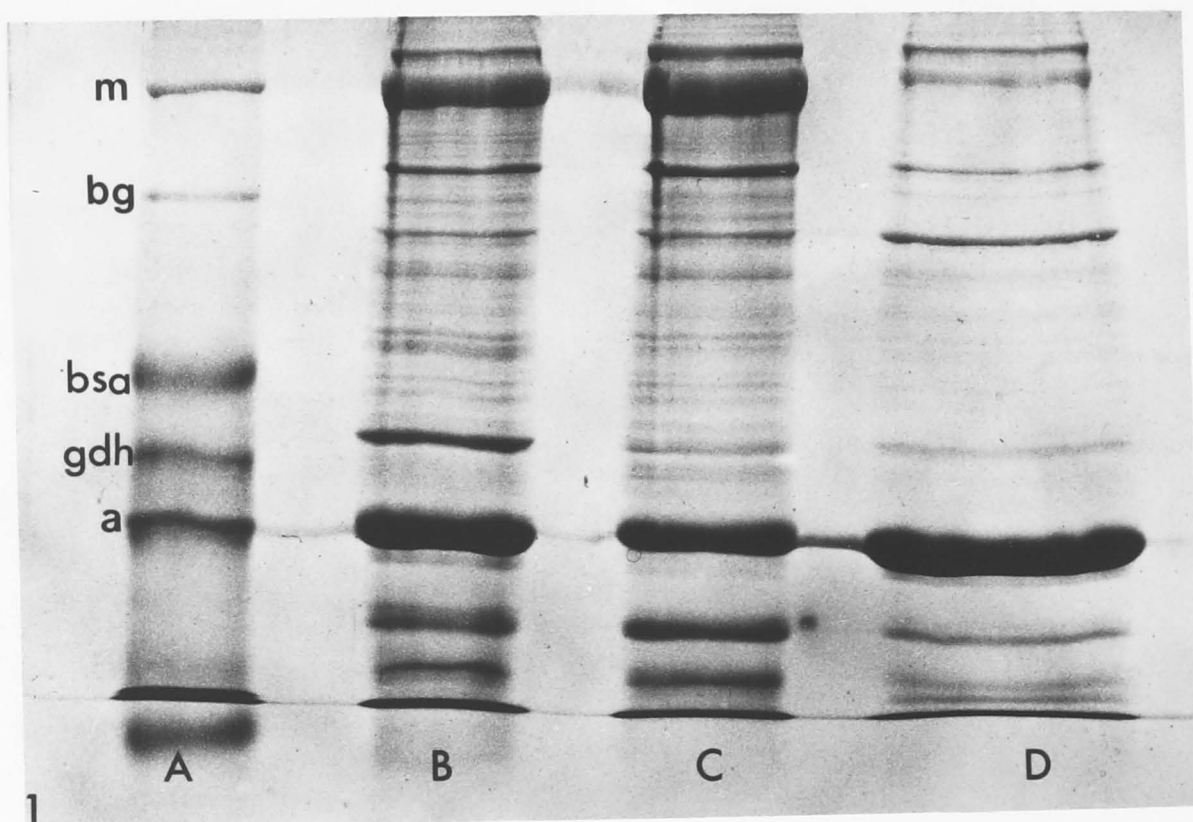
(a) *Isolation of intermediate filaments:* The procedure used for the isolation of intermediate filaments from chicken gizzard (for details, see Chapter 2, P.44) is a modification of the method used by Small and Sobieszek (1977). The low ionic strength extraction step used by these workers was found to be redundant. Repeated extractions using high ionic strength (0.6M KCl) actomyosin extracting solutions (HAMES) containing either 5mM ATP or 30mM pyrophosphate were found to be effective in extracting the actomyosin.

Fig. 1 is an SDS polyacrylamide gel electrophoresis profile illustrating the presence of actin and myosin in the supernatant after extraction by HAMES. The supernatants of initial extractions were analysed ultrastructurally. As illustrated in Fig. 2, numerous

Fig. 1: SDS polyacrylamide (10%) gel electrophoretic profile showing proteins present in initial stages in the isolation of intermediate filaments (for methods, see Chapter 2, p.44).

- (A) Protein stands: These are - purified skeletal muscle myosin (m) (200K),  $\beta$ -galactosidase (bg) (130K), bovine serum albumin (bsa) (68K), glutamate dehydrogenase (gdh) (53K), and actin (a).
- (B) Homogenate of chicken gizzard smooth muscle in standard salt solution showing two prominent bands corresponding to myosin (m) (200K) and actin (a) (42K). Other bands of significant quantities correspond to molecular weights of 55K, 220K, 140K, 118K and 30K. Many minor bands are also present.
- (C and D) Extract obtained after HAMES (high ionic strength actomyosin extracting solution) extraction showing a large quantity of actin, and smaller but significant quantities of myosin and proteins other than the 55K protein. As demonstrated here, very little 55K protein is extracted by HAMES.

Fig. 2: An electron micrograph showing a negatively stained preparation of an extract obtained after HAMES extraction. Short actin filaments can be seen decorated by monomers of myosin resulting in an arrowhead configuration. No myosin thick filaments are seen. X 88,000.





short actin filaments are decorated by myosin molecules, resulting in 'arrowhead' formations. No myosin thick filaments can be seen.

After repeated HAMES extractions, the residue was found to be highly enriched in intermediate filaments. No filaments resembling either thick myosin or thin actin filaments remained in association with the intermediate filaments. However, the SDS gel derived from this intermediate filament preparation shows, in addition to large amounts of 55K protein, the presence of significant quantities of actin (Fig. 3). A protein corresponding to the molecular weight of myosin and a few minor bands are present as well.

(b) *Ultrastructure of intermediate filaments:* As shown in Fig. 4, intermediate filaments occur as aggregates consisting of large numbers of long curvilinear filaments. Collagen fibres are occasionally associated with these aggregates. A high power view (Fig. 5) indicates that these filaments are relatively uniform in diameter, averaging 11 nm (range 10-12 nm). This is consistent with the finding of Small and Sobieszek (1977). Each intermediate filament appears to consist of 4-5 sub-filaments. Small and Squire (1972) reported finding 4 sub-filaments in the intermediate filaments of sectioned chicken gizzard smooth muscle cells.

(c) *Effect of DNAase I on intermediate filaments:* Intermediate filaments were treated with DNAase I under the same conditions as used in the case of cultured chick

Fig. 3: SDS polyacrylamide (10%) gel electrophoretic profile of a residue after several HAMES extractions shows abundant quantities of 55K protein, a small but significant quantity of actin, a slightly smaller quantity of a 200K protein and traces of a 220K protein. Also present are some faint unidentified bands. A, B, C and D correspond to varying amounts of residue applied to the gel. E shows protein standards which consist of purified skeletal muscle myosin (m) (200K),  $\beta$ -galactosidase (bg) (130K), bovine serum albumin (bsa) (68K), glutamate dehydrogenase (gdh) (53K), and purified skeletal muscle actin (a). The lowest band is that of myosin light chain (lc).

Fig. 4: Electron micrograph of a negatively stained preparation of intermediate filaments isolated from chicken gizzard smooth muscle. The intermediate filaments are more or less uniform in diameter ( $\sim 11$  nm), long and intertwined, sometimes occurring in clumps. Thin actin filaments or thick myosin filaments are not seen in this preparation. X 75,000.

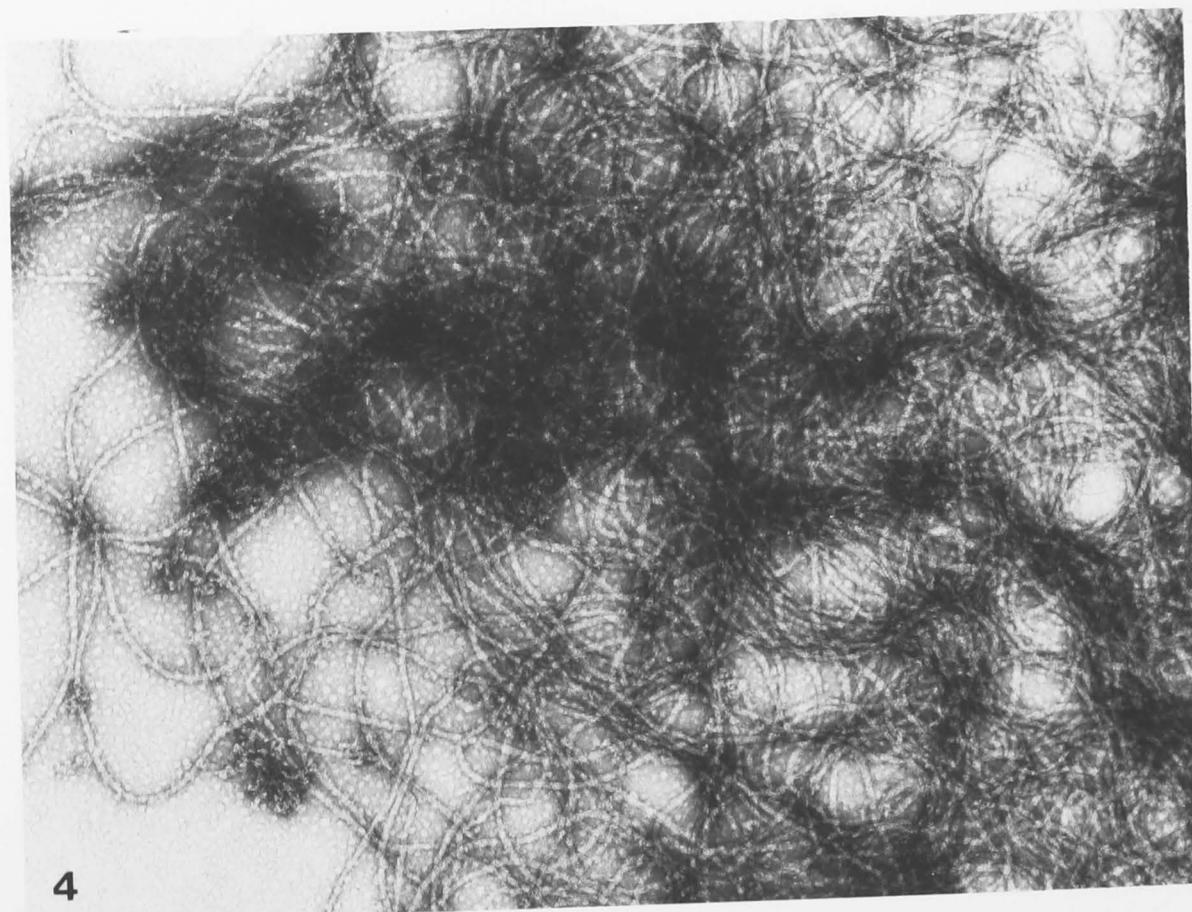
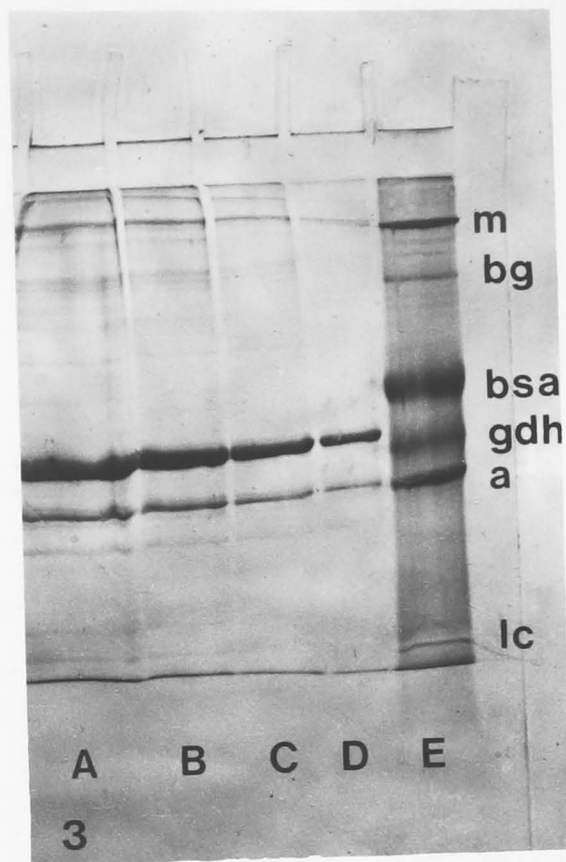
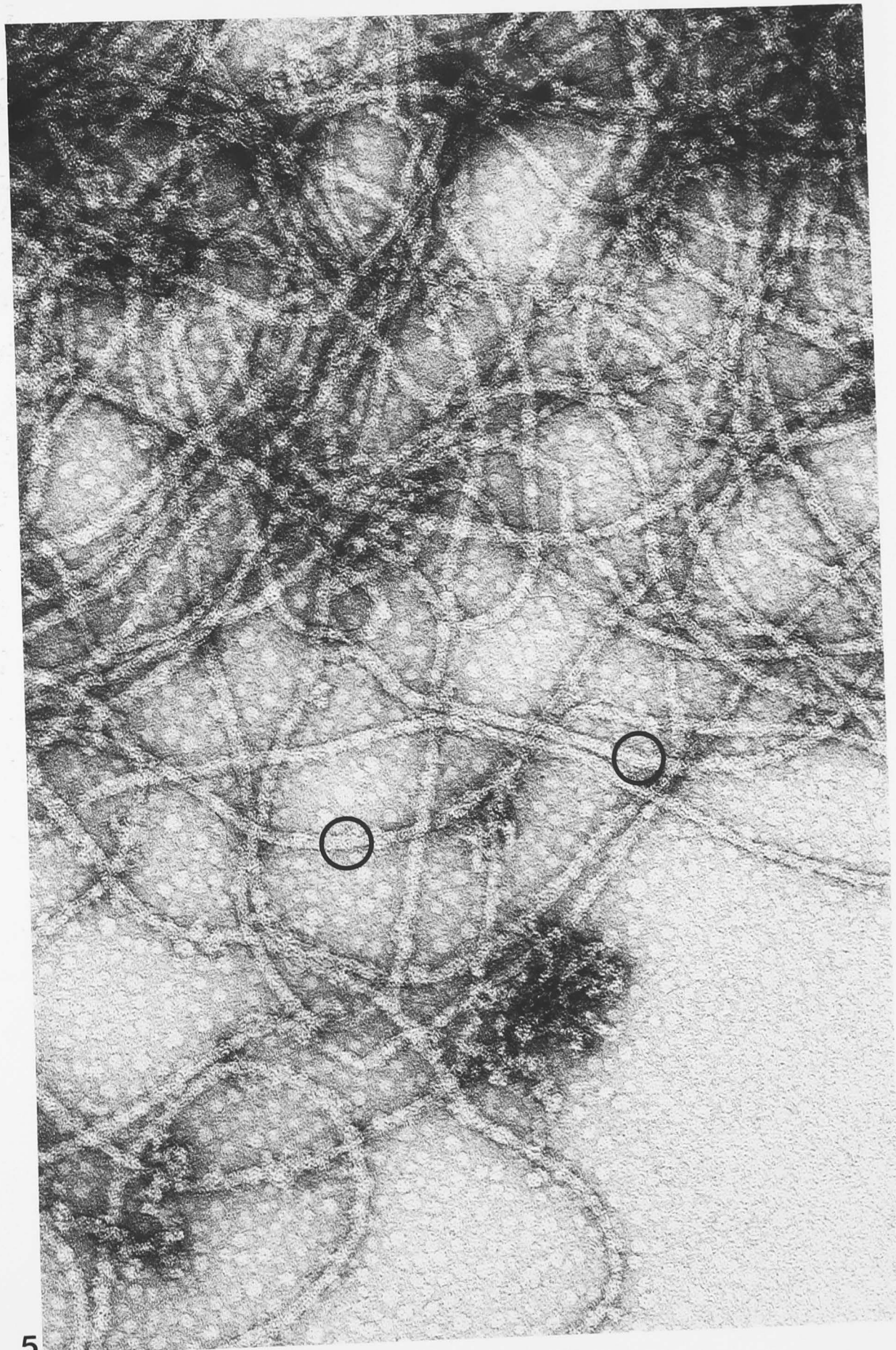


Fig. 5: A high power electron micrograph of isolated intermediate filaments reveals that they contain 4 subfilaments (circled areas). As in the previous micrograph, no thin filaments can be observed. X 220,000.





embryo connective tissue cells. Fig. 6 represents a control electron micrograph of filaments which were not subjected to DNAase I treatment but where all other conditions were common to both the control and the DNAase I-treated filaments. A comparison of Figs. 6 (control) and 7, which shows the DNAase I-treated intermediate filaments, illustrates that the DNAase I treatment has not resulted in any discernible change in the structure of these filaments. This finding contrasts with what occurred in the cultured chick embryo connective tissue cells, where intermediate filaments were depolymerized, along with the thin filaments (see Chapter 5).

*(d) Effect of trypsin and HMM on intermediate filaments:*

Intermediate filaments of chicken gizzard did not label with HMM under conditions which allowed the labelling of thin actin filaments. Earlier it was shown (see Chapter 4) that in cultured chick embryo connective tissue cells, HMM labelled intermediate filaments, after a gentle trypsin treatment which did not eliminate them. This indicated that a trypsin-resistant F-actin core forms the basis of intermediate filaments in cultured fibroblasts. However, intermediate filaments isolated from chicken gizzard were completely destroyed by this same gentle trypsin treatment.

Fig. 8 is an electron micrograph of a control preparation, i.e. one not treated with trypsin. Here, the intermediate filaments are long and take a curvilinear course. Collagen fibres can be seen associated with the intermediate filaments. After gentle trypsin treatment (5 $\mu$ g/ml for 1-2 hours at room temperature), the intermediate

Fig. 6: Control for DNAase I experiment for which an intermediate filament preparation was subjected to a change in the ionic environment and standing at room temperature but not DNAase I (for details of treatment, see Chapter 2, p.46).

This micrograph shows that standing at room temperature and in a reduced ionic strength solution (from 0.6M to 0.1M KCl) for 2 hours has no appreciable effect on the structure of intermediate filaments. X 70,000.

Fig. 7: This electron micrograph shows a preparation of intermediate filaments which were subjected to DNAase I treatment (for methods, see Chapter 2, p.46). As the micrograph illustrates, DNAase I has no discernible effect on the structure of these intermediate filaments. Other conditions were the same as in the control (cf. Fig. 6). X 50,000.



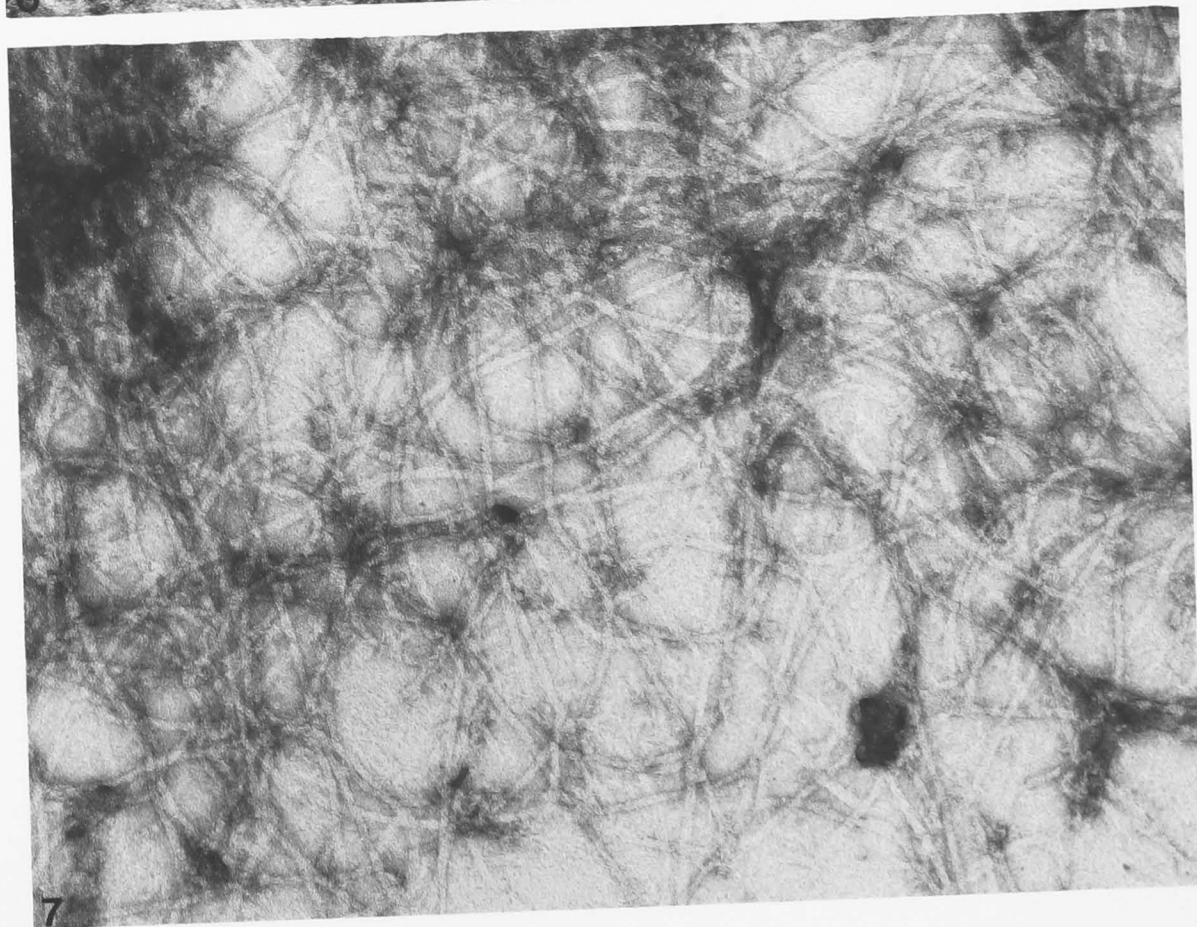
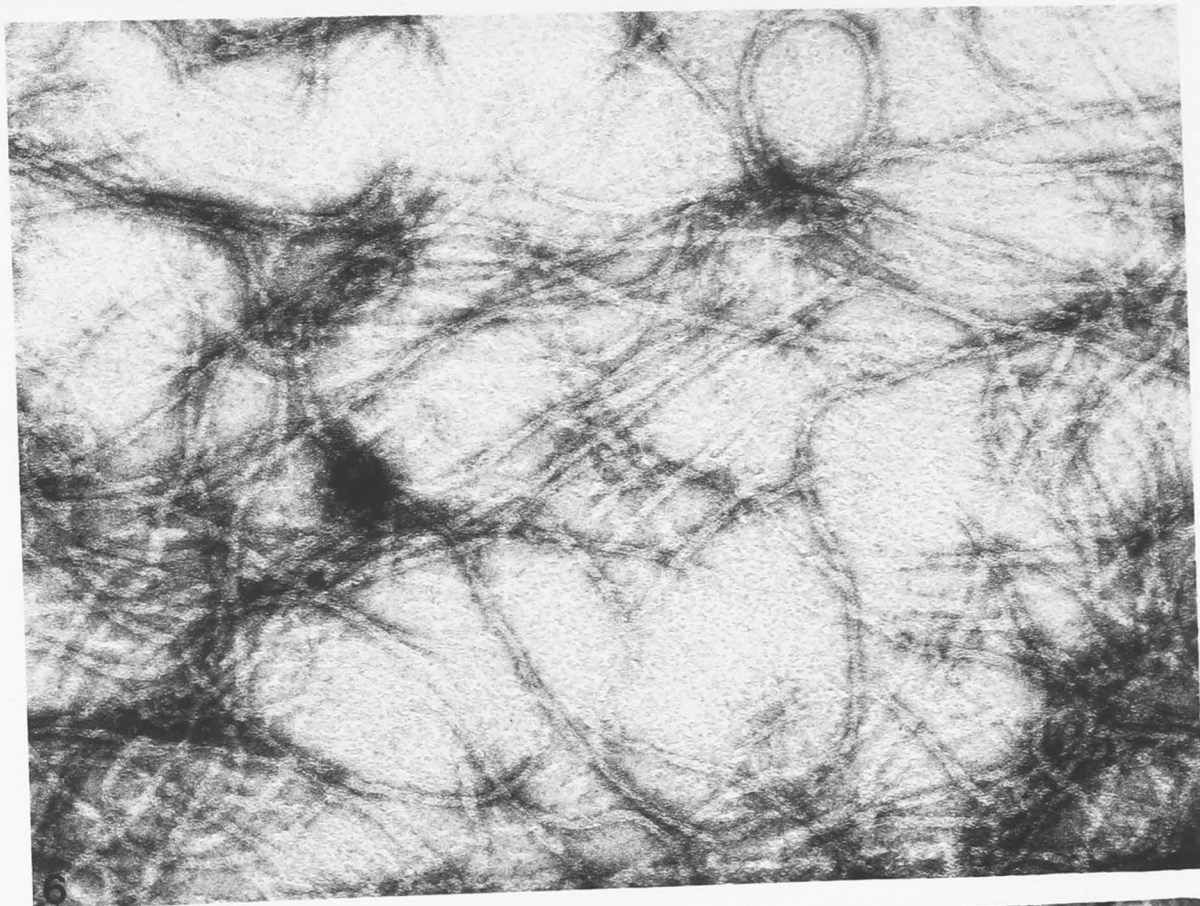
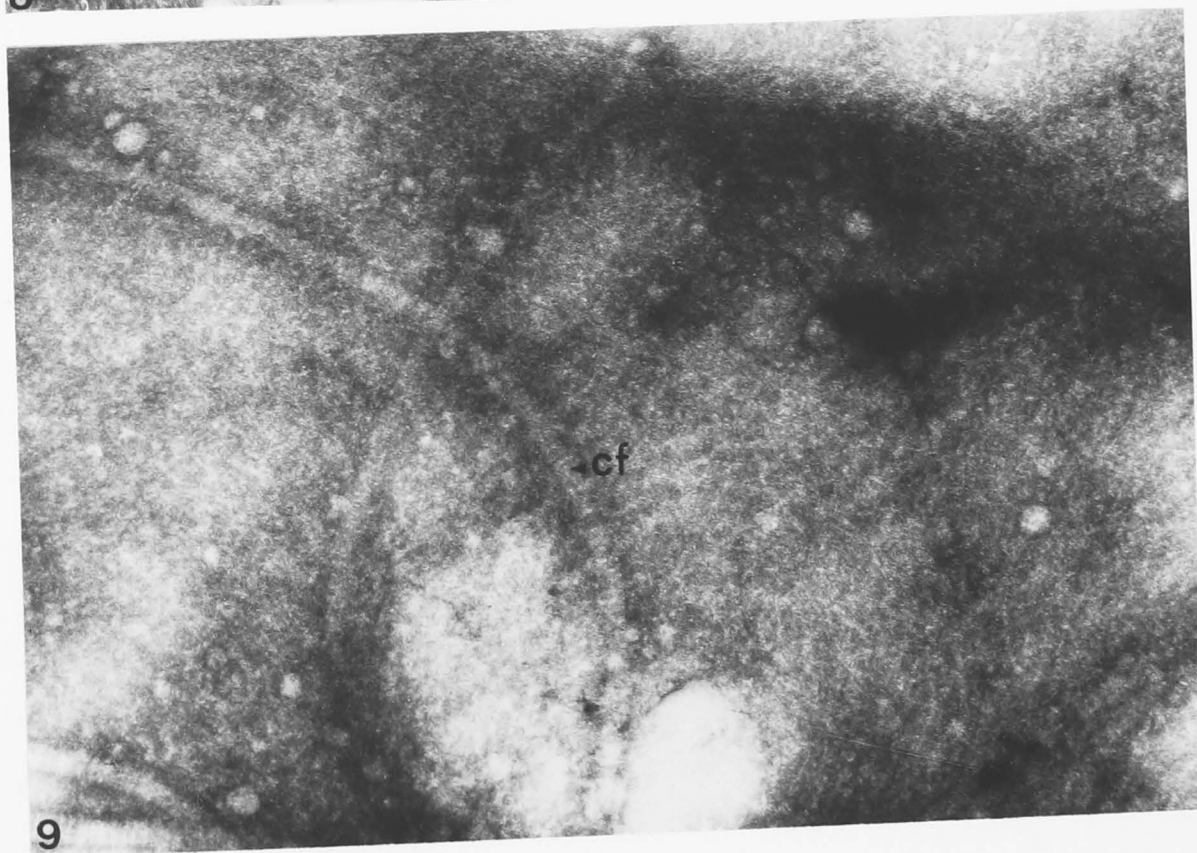
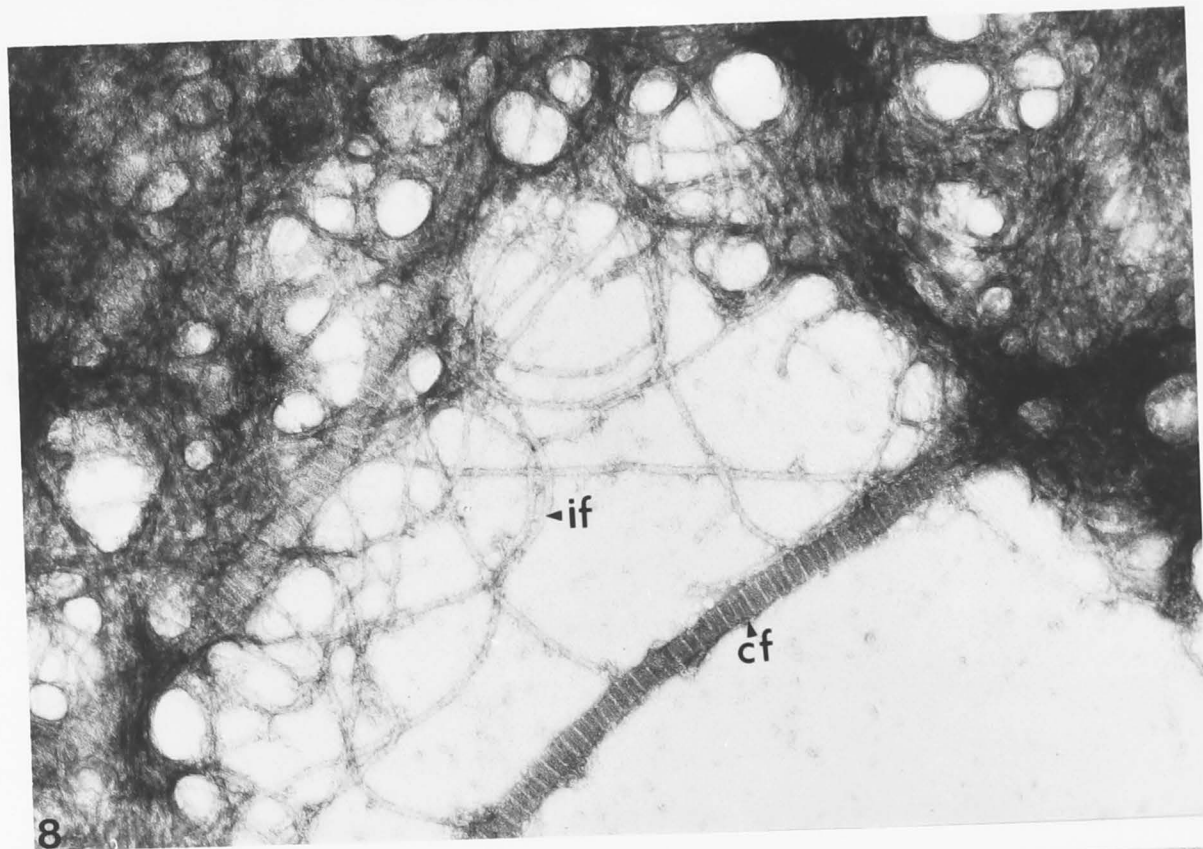




Fig. 8: Control for trypsin experiment (for methods, see Chapter 2, p.46). The electron micrograph shows intermediate filaments (if) some occurring in clumps. Collagen fibres (cf), as identified by their cross-banding, can be seen associated with the intermediate filaments. X 90,000.

Fig. 9: Electron micrograph showing negatively stained product obtained after gentle trypsin treatment of intermediate filaments. When intermediate filaments were given a gentle trypsin treatment to see whether they would then label with HMM or not, they were completely destroyed. The collagen fibres (cf) were not disrupted by this trypsin treatment. X 88,000.



filaments are completely lost and only electron-dense amorphous material is seen (Fig. 9). In this same micrograph collagen fibres appear to remain unaffected.

(e) *Effect of aqueous extraction on the structure of intermediate filaments:* In an attempt to obtain 55K protein free of actin, the residue after HAMES extraction was again extracted repetitively with buffered glass-distilled water. As the gel (Fig. 10) indicates, a large quantity of actin is present in the supernatants after water extraction. However, the most surprising result was the disruption of the structure of intermediate filaments. This was revealed when the residue after water extraction was viewed under the electron microscope. As shown in Fig. 11, the filaments are broken up at several places and only densely staining globular aggregates remain. The extent of the breakage of filaments increased progressively after each water extraction.

#### DISCUSSION

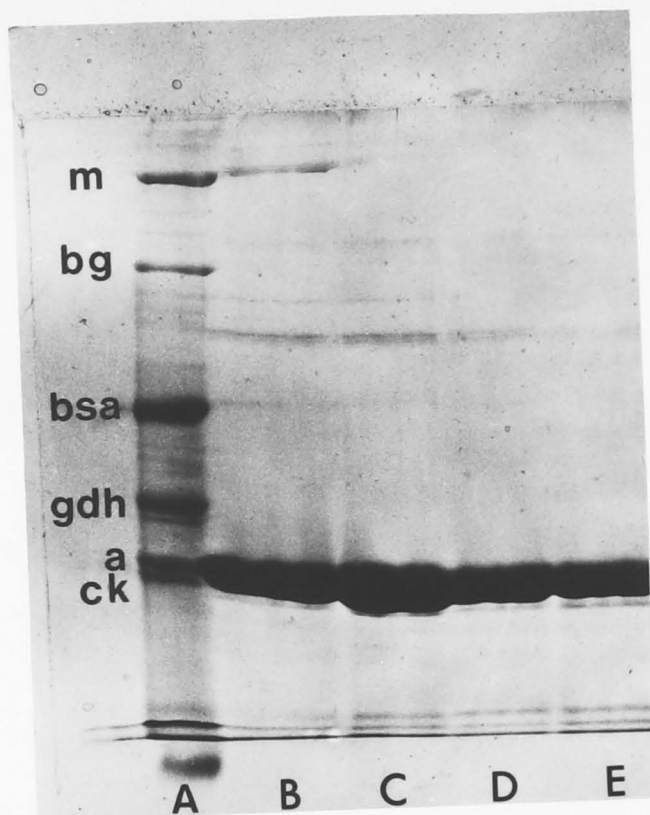
The results of the experiments described above can be summarized as follows:

- (1) DNAase I does not depolymerize the intermediate filaments of chicken gizzard smooth muscle;
- (2) The filaments can not be made to label with HMM: the gentle trypsin pre-treatment (which made possible the labelling of intermediate filaments of chick embryo connective tissue cells) completely destroys the intermediate

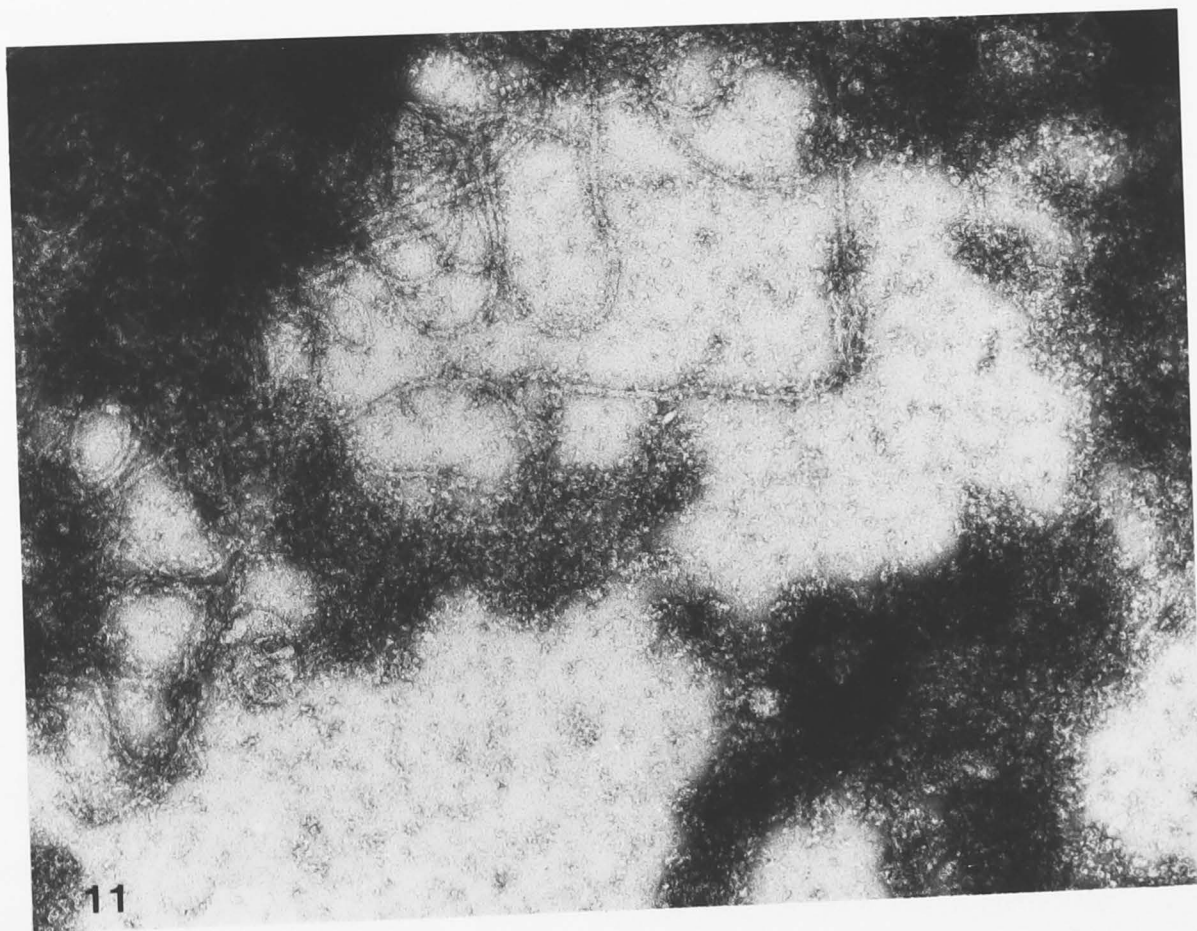
Fig.10: SDS polyacrylamide gel (10%) electrophoretic profile of extracts (B, C, D and E) obtained after distilled water extraction. This gel illustrates that actin is preferentially extracted, by this procedure, from preparations rich in intermediate filaments. A refers to standards consisting of myosin (m) (200K),  $\beta$ -galactosidase (bg) (130K), bovine serum albumin (bsa) (68K), glutamate dehydrogenase (gdh) (53K), actin (a) (42K) and creatine kinase (ck) (40K).

Fig.11: Electron micrograph showing a negatively stained preparation of intermediate filaments which were subjected to distilled water extraction. The intermediate filaments are disrupted by this procedure. Accumulation of electron dense material formed by the disruption of filaments are seen.  
X 75,000.





10



11

filaments of chicken gizzard.

(3) In intermediate filament preparations which, by electron microscopy, appear homogenous (free of any thin actin or thick myosin filaments) SDS gels reveal significant quantities of actin along with another major (55K) protein and several minor proteins.

(4) When intermediate filament preparations are treated with buffered, glass-distilled water, the filaments are progressively broken down, this being accompanied by a gradual extraction of actin.

The above-mentioned results indicate that F-actin, which forms the core of intermediate filaments of chick embryo connective tissue cells (see Chapters 4 and 5), is not present in the intermediate filaments of chicken gizzard. This is convincingly demonstrated by both the DNAase I and trypsin-HMM labelling experiments. In the case of chick embryo connective tissue cells, the DNAase I treatment (by depolmerization of the F-actin core of the intermediate filaments), resulted in the complete loss of these filaments. This appears to demonstrate that there is an F-actin core which is critical for maintaining the structure of the intermediate filaments of chick embryo connective tissue cells. However, in the case of chicken gizzard smooth muscle intermediate filaments, DNAase I treatment produces no detectable fine structural change and this appears to indicate that in these intermediate filaments, there is no structure-maintaining F-actin core.

This interpretation is supported by the experiments which were designed to label the chicken gizzard intermediate filaments with HMM after a gentle trypsin pre-treatment. In contrast to the findings with chick embryo connective tissue cells (Buckley, Raju and Stewart, 1978; see also Chapter 4), where this type of pre-treatment enabled the subsequent HMM labelling of their intermediate filaments, the effect of this gentle trypsinization on the intermediate filaments of chicken gizzard was to completely destroy them. As actin in the F-form is known to resist such trypsin treatment, the above result appears to show clearly that F-actin is not present within the intermediate filaments of chicken gizzard.

Assuming that actin is not present in the filamentous form (i.e. as F-actin) in chicken gizzard intermediate filaments, is there any possibility of it being present in some non-filamentous form? The presence of actin in the intermediate filament preparation as shown by the SDS gel results described above, was also observed by other workers (Cooke, 1976; Lazarides and Hubbard, 1976; Small and Sobieszek, 1977). While Cooke (1976) and Small and Sobieszek (1977) interpreted the presence of actin as due to contamination, Lazarides and Hubbard (1976) considered the possibility of actin being a constituent of intermediate filaments. Later, Lazarides and Balzer (1977) found additional evidence for the occurrence of actin as an integral component of the intermediate filaments of chicken smooth muscle. When they analysed the intermediate filament actin fraction by two dimensional gel electrophoresis, they found it to be exclusively a

$\gamma$ -variant of actin, in contrast to the actin in the initially extracted acto-myosin, which was all  $\beta$ -variant. The present results support the work of Lazarides and Hubbard (1976) and Lazarides and Balzer (1977).

It is hard to agree with the claim of Small and Sobieszek (1977) that the intermediate filaments of chicken gizzard do not contain actin. The reasons for doubting their claim are as follows:

- (1) In their procedure for obtaining purified intermediate filaments, actin continued to co-purify with the 55K protein until harsh measures (such as urea solubilization and acetic acid extraction) were applied. These harsh measures could have been deleterious to actin.
- (2) The filaments reconstituted by these authors using purified 55K protein under non-physiological conditions (such as using a pH of 2-4), do not resemble the native intermediate filaments.

The possible functional significance of the presence of F-actin in the intermediate filaments of chick embryo connective tissue cells on the one hand and the absence of F-actin from the intermediate filaments of chicken gizzard on the other should be considered. It may be that intermediate filaments of chick embryo connective tissue cells play a dual role, first as a cyto-skeletal structure-determining physical support to the cell body and secondly as a structure providing one component of a shear force-generating apparatus for organelle movements through the sub-cortical cytoplasm. An underlying F-actin core in these filaments could help to achieve both roles. The intermediate filaments of chicken



gizzard smooth muscle, however, may act simply as a cyto-skeletal structure, preventing excessive localized distensions which might otherwise result in rupture of the cells during contraction.

The major intermediate filament-specific protein of non-muscle cells, such as fibroblasts, has a similar molecular weight, ~55K, (Starger *et al.*, 1978) to that of smooth muscle. Nevertheless, it is still uncertain whether these proteins are related. Campbell *et al.* (1978) found that fluorescein-labelled anti-bodies raised against the SDS-denatured 55K protein isolated from chicken gizzard intermediate filaments did not stain the intermediate filaments of cultured fibroblasts. However, Kurki *et al.* (1977) found that the anti-bodies which stain the intermediate filaments of non-muscle cells, such as human embryonic skin fibroblasts, also cross react with a preparation of intermediate filaments purified from chicken gizzard according to the method of Cooke (1976). This particular intermediate filament preparation is highly enriched in 55K protein (Cooke, 1976). Consequently, the relationship between the 55K protein of the smooth muscle intermediate filaments and the 55K protein of non-muscle cell intermediate filaments has yet to be determined. Nevertheless, it seems conceivable that the major intermediate filament protein of chicken gizzard smooth muscle could be related to its counterpart in at least some of the non-muscle cells (Starger *et al.*, 1978; Franke *et al.*, 1978).

Future work in the following areas will throw more light on the structure of intermediate filaments of chicken

gizzard smooth muscle:

- (1) Studies should be aimed at determining whether 55K protein, isolated by gentle methods from chicken gizzard smooth muscle, can alone self-assemble to form intermediate filaments under physiological conditions.
- (2) If 55K protein alone does not self-assemble, actin must be also added to the 55K preparation to see whether assembly of intermediate filaments is then possible under physiological conditions. This actin must be obtained from a morphologically pure intermediate filament preparation obtained after extensive actomyosin extraction.
- (3) If intermediate filaments can be synthesized in vitro, they must be carefully compared with the intermediate filaments occurring in the cell by electron microscopic and optical diffraction analysis.
- (4) Finally, biochemical studies should be carried out to determine the functional significance of the  $\gamma$ -variant of actin.

The aim of this work has been to increase understanding of how non-muscle cells move, by defining the form and distribution of F-actin and myosin in cultured chick embryo connective tissue cells. This problem has been approached by combining light and electron microscopy with a variety of cytochemical and biochemical techniques.

The results of this thesis can be summarized as follows:

#### Structures Associated with Moving Parts of the Cell

### CHAPTER 8 - SUMMARY AND CONCLUSIONS

The villi-like and microvilli extensions of the cell surface and the organelles in the deeper cytoplasm, show a constant close association with filaments. This filaments, 4-7 nm in diameter, are closely associated with movements of the cell surface and intermediate filaments, 7-11 nm in diameter, are associated with the movements of organelles through the sub-cortical cytoplasm. All of these filaments have attached to them, numerous 'tail-like' structures with fine tails. Frequently these, joined tail to tail, form 'dumb-bell' structures which cross-link filaments to one another, to microtubules and to nearby organelles. Having defined the fine structure of the cell's filament system in relation to its moving parts, attempts were made to identify which of its components contain filamentous proteins and which represent cytoplasmic myosin.

The aim of this work has been to increase understanding of how non-muscle cells move, by defining the form and distribution of F-actin and myosin in cultured chick embryo connective tissue cells. This problem has been approached by combining light and electron microscopy with a variety of cytochemical and biochemical techniques.

The results of this thesis can be summarized as follows:

#### Structures Associated with Moving Parts of the Cell

The actively moving parts of living cells, notably the veil-like and microvillus extensions of the cell surface and the organelles in the deeper cytoplasm, show a constant close association with filaments. Thin filaments, 4-7 nm in diameter, are closely associated with movements of the cell surface and intermediate filaments, 7-11 nm in diameter, are associated with the movements of organelles through the sub-cortical cytoplasm. All of these filaments have attached to them, numerous tiny 'node-like' structures with fine tails. Frequently these, joined tail to tail, form 'dumb-bell' structures which cross-link filaments to one another, to microtubules and to nearby organelles. Having defined the fine structure of the cell's filament system in relation to its moving parts, attempts were made to identify which of its components contain filamentous actin and which represent cytoplasmic myosin.



### F-actin Localization

To determine the localization of F-actin, studies were made with both heavy meromyosin (HMM), a tryptic fragment of muscle myosin known to bind to F-actin to form arrowhead complexes, and DNAase I which specifically couples to F-actin and depolymerizes it. In common with the experience of previous investigators, in glycerinated/myosin-extracted cells treated with HMM, only the thin filaments of the sub-plasmalemmal cytoplasm and cell cortex labelled to form arrowhead patterns. The predominantly intermediate-sized filaments of the sub-cortical cytoplasm failed to label. Exploring the possibility that this failure to label could be due to the blocking effect of some accessory proteins, advantage was taken of the known relative resistance of F-actin to trypsin to see whether its action might unmask underlying F-actin. When glycerinated/myosin-extracted cells were given a controlled trypsin treatment before the application of HMM, virtually all filaments showed clear arrowhead patterns, most notably the intermediate-sized ones which had previously failed to label. As control experiments demonstrated that these intermediate filaments were not destroyed by the trypsin treatment, it could be concluded that the intermediate filaments of the sub-cortical cytoplasm, as well as the thin filaments of the cell cortex, contained filamentous actin.

The glycerinated/myosin-extracted cultured chick embryo connective tissue cells were also treated with DNAase I, which is known to selectively bind to muscle F-actin and completely depolymerize it by a non-enzymic

action. The results obtained from the cells treated with DNAase I showed that not only did the thin filaments undergo depolymerization, but so also did the intermediate filaments. Accordingly, these results support the idea that intermediate as well as thin filaments contain F-actin. Therefore, these results support the conclusion drawn from the HMM labelling experiments.

#### Myosin Localization

Electron microscopic examination of normal (unextracted) cultured connective tissue cells failed to reveal any structures resembling myosin thick filaments.

As no myosin-specific marker suitable for electron microscopy was available, evidence for the identification of cytoplasmic myosin was less direct than for actin. Using myosin-extracting solutions to treat glycerinated cells, attempts were made to determine which structures were removed by this treatment and whether this was accompanied by the appearance of myosin in the extracts, as determined by SDS gel electrophoresis.

When glycerinated cells were treated with pyrophosphate-based myosin-extracting solutions, the 'node-like' and 'dumb-bell' shaped structures were removed, leaving behind smooth residual filaments. This change was accompanied by the appearance in the extract of bands which comigrated with myosin heavy chains. All other bands in the extract appeared essentially as in the extract obtained by extraction with glycerol/standard salt solution (which did not have any pyrophosphate).

This suggested the possibility that a high proportion of the 'node-like' and 'dumb-bell' shaped structures represent cytoplasmic myosin.

To explore this idea, model experiments were carried out with purified actin and myosin derived from skeletal muscle. When F-actin and myosin were mixed together in a molar ratio approximating that of non-muscle cells (i.e.  $\sim 50:1$ ) then, even under conditions known to favour the formation of myosin thick filaments, the monomeric myosin preferentially bound to the actin filaments instead of self-aggregating. The resulting actin myosin filamentous complex bore a clear resemblance to the filamentous system of intact cultured cells.

Accordingly, it is tentatively concluded, that many of the 'node-like' and 'dumb-bell' shaped structures found in intact and glycerinated cultured cells represent cytoplasmic myosin in some highly disperse form, possibly as monomers and dimers.

#### Intermediate Filaments of Smooth Muscle

Isolated preparations of the intermediate filaments of chicken gizzard smooth muscle were investigated to see whether these too might contain F-actin. However, in contrast to the intermediate filaments of chick embryo connective tissue cells, when these smooth muscle intermediate filaments were given the trypsin treatment, they were rapidly destroyed and when treated with DNAase I, they appeared to remain unaffected. At the same time, as shown by SDS gel electrophoretic analyses, smooth muscle intermediate filament preparations, which

ADDENDUM

## Proposed Models

There is no experimental evidence to prove that proposed model (i) is the functioning system in non-muscle cell movements. However, the structural evidence as reported in this thesis shows that the 'node-like' structures are thought to be myosin molecules whose tails cross-link adjacent actin filaments. Korn (1978) has also proposed a similar model to explain cell motility. He further stated that this model satisfies the requirement of a sliding filament mechanism as described for skeletal muscle contraction. Accordingly, even though the proposed model (i) is lacking experimental evidence at this stage, it is worthwhile pursuing. Whether or not any auxillary protein is involved in linking myosin tails to actin filaments is unknown.

The proposed model (ii) states that myosin in dimeric form, by cross-linking two actin filaments, can bring about relative movements of filaments. Such a model has already been described in detail by Panner and Honig (1967)\* in smooth muscle. These authors also showed that their model satisfied the 'length-tension' relationship of smooth muscle contraction. Nachmias (1972) also proposed a similar model to explain motility in *Physarum*. Hence, proposed model (ii) is thought to be functionally meaningful.

With regard to proposed model (iii) the evidence for interaction between actin, myosin and mitochondria is based purely on morphological observations (Buckley and Raju, 1976; see also Chapter 3 in this thesis). The mitochondria are surrounded by a three dimensional network of filaments, which contain actin. It was also observed that short 'dumb-bell' shaped structures cross-link mitochondria with the network of actin filaments. However, it must be stated here that biochemical evidence such as localization of myosin or actin on the outer membrane of mitochondria must be obtained to support this model. Another possible experiment would be to isolate mitochondria from cells and coat them with non-muscle myosin. The next step would be to introduce the myosin coated mitochondria into a Rose Chamber where the glass coverslip is coated with polymerized actin filaments. If the proposed model (iii) is correct, the mitochondria should exhibit movements in the presence of suitable ionic strengths and ATP.

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\* PANNER, B.J. and HONIG, C.R. (1967).

Filament ultrastructure and organization in vertebrate smooth muscle: contraction hypothesis based on localization of actin and myosin. J. Cell Biol., 35, 303-321.



appeared to be free of thin filaments, nevertheless contained significant quantities of actin. From this it was tentatively concluded that actin is incorporated in the structure of these intermediate filaments, but in some non-filamentous form.

#### Proposed Model

Based on the results obtained with the structure and localization of F-actin and myosin in cultured chick embryo connective tissue cells, it is proposed that movements in non-muscle cells may be brought about by one or more of the following mechanisms.

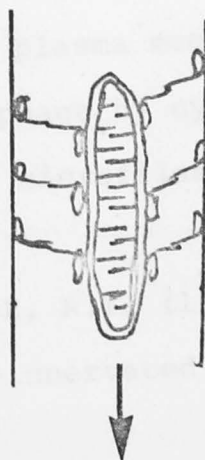
- (i) Interaction between two F-actin filaments through monomers of myosin molecules: It is conceivable that individual myosin molecules might attach to an F-actin filament through their tails. Then the heads which are free could interact with another F-actin filament, resulting in the movement of the latter in relation to the former.



- (ii) Interaction between two F-actin filaments through dimers of myosin:  
The heads of myosin dimers might interact with two F-actin filaments polarized in opposite directions, to bring about relative movement.



- (iii) Organelle movements: Organelles might be moved, by interacting with F-actin containing intermediate filaments, through attached monomers or dimers of myosin.



## REFERENCES

- ADELSTEIN, R.S., CONTI, M.A. JOHNSON, G.S. PASTAN, I. and POLLARD, T.D. (1972) Isolation and characterization of myosin from cloned mouse fibroblasts. Proc. Natn. Acad. Sci. U.S.A., 69, 3693-3697.
- ALLISON, A.C., DAVIES, P. and de PETRIS, S. (1971) Role of contractile microfilaments in macrophage movement and endocytosis. Nature New Biol., 232, 153-155.
- AMOS, W.B. (1978) Contraction and calcium binding in the vorticellid spasmoneme. In Abstracts of sixth International Biophysics Congress, p. 58, International Union for Pure and Applied Physics, Kyoto, Japan.
- ANDERSON, T.F. (1951) Techniques for the preservation of three-dimensional structure in preparing specimens for the electron microscope. Trans, N.Y. Acad. Sci., Series II, 13, 130-135.
- ARRONET, N.I. (1973) Motile muscle and cell models. Consultants Bureau, New York.
- AXLINE, S.G. and REAVEN, E.P. (1974) Inhibition of phagocytosis and plasma membrane mobility of the cultivated macrophage by cytochalasin B. Role of sub-plasmalemmal microfilaments. J. Cell Biol. 62, 647-659.
- BÁRÁNY, M. and CLOSE, R.I. (1971) The transformation of myosin in cross-innervated rat muscles. J. Physiol. 213, 455-474.
- BEHNKE, O., KRISTENSEN, B.I. and NIELSEN, L.E. (1971) Electron microscopical observations on actinoid and myosinoid filaments in blood platelets. J. Ultrastruct. Res., 37, 351-369.

- BERENBLUM, I. (1964) The epidemiology of cancer. In General Pathology (Third edition) (Ed. by H. Florey) p.598, Lloyd-Leuke (Medical Books) Ltd., London.
- BERGER, G. and MAY, P. (1967) Purification et proprietes d'un inhibiteur de la desoxyribonuclease pancreatique, extrait du serum de rat. Biochim. Biophys. Acta, 139, 148-161.
- BETTEX-GALLAND, M. PORTZEHL, H. and LÜSCHER, E.F. (1962) Dissociation of thrombosthenin into two components comparable with actin and myosin. Nature, 193, 777-778.
- BLOSE, S.H., SHELANSKI, M.L. and CHACKO, S. (1977) Localization of bovine brain filament antibody on intermediate (100Å) filaments in guinea pig vascular endothelial cells and chick cardiac muscle cells. Proc. Natn. Acad. Sci. U.S.A., 74, 662-665.
- BRAY, D. and THOMAS C. (1975) The actin content of fibroblasts. Biochem. J., 147, 221-228.
- BRAY, D. and THOMAS, C. (1976) Unpolymerized actin in fibroblasts and brain. J. Mol. Biol., 105, 527-544.
- BUCKLEY, I.K. (1964) Phase contrast observations on the endoplasmic reticulum of living cells in culture. Protoplasma, 59, 569-588.
- BUCKLEY, I.K. (1973) Studies in fixation for electron microscopy using cultured cells. Lab. Invest., 29, 398-410.
- BUCKLEY, I.K. (1974) Subcellular motility: a correlated light and electron microscopic study using cultured cells. Tissue Cell, 6, 1-20



- BUCKLEY, I.K. (1975) Three dimensional fine structure of cultured cells, possible implication for subcellular motility. Tissue Cell, 7, 51-72.
- BUCKLEY, I.K. and PORTER, K.R. (1967) Cytoplasmic fibrils in living cultured cells. A light and electron microscope study. Protoplasma, 64, 349-380.
- BUCKLEY, I.K. and PORTER, K.R. (1973) Advances in electron microscopy of whole cells grown in vitro. J. Cell Biol., 59, 37a.
- BUCKLEY, I.K. and PORTER, K.R. (1975) Electron microscopy of critical-point dried whole cultured cells. J. Microsc., 104, 107-120.
- BUCKLEY, I.K. and RAJU, T.R. (1976) Form and distribution of actin and myosin in non-muscle cells: a study using cultured chick embryo fibroblasts. J. Microsc., 107, 129-149.
- BUCKLEY, I.K., RAJU, T.R. and STEWART, M. (1977) Some intermediate-sized filaments in cultured chick embryo fibroblasts contain F-actin. J. Cell Biol., 75, 257a.
- BUCKLEY, I.K., RAJU, T.R. and STEWART, M. (1978) Heavy meromyosin labelling of intermediate filaments in cultured connective tissue cells. J. Cell Biol., 78, 644-653.
- BURRIDGE, K. (1976) Multiple forms of non-muscle myosin. In Cell Motility (Book B) (Ed. R. Goldman, T. Pollard and J. Rosenbaum) p.739, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- BURRIDGE, K. and BRAY, D. (1975) Purification and structural analysis of myosins from brain and other non-muscle tissues. J. Mol. Biol., 99, 1-14.

- CAMPBELL, G.R., CHAMLEY-CAMPBELL, J.H., GROSCHEL-STEWART, U., SMALL, J.V. and ANDERSON, P. (1978) Immunofluorescent localization of 10 nm (100<sup>o</sup>Å) filaments in muscle and non-muscle cells. In Abstracts of papers presented at the Meeting: Cytoskeletal and Contractile Networks of Non-muscle Cells, p.43, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- CLARKE, M. and SPUDICH, J.A. (1977) Non-muscle contractile proteins: the role of actin and myosin in cell motility and shape determination. Ann. Rev. Biochem., 46, 797-822.
- COOKE, P.H. (1976) A filamentous cytoskeleton in vertebrate smooth muscle fibres. J. Cell Biol., 68, 539-556.
- COOKE, P.H. and CHASE, R.H. (1971) Potassium chloride-insoluble myofilaments in vertebrate smooth muscle cells. Exp. Cell Res., 66, 417-425.
- COOKE, R., CLARKE, M., von WEDEL, R.J. and SPUDICH, J.A. (1976) Supramolecular forms of Dictyostelium actin. In Cell Motility (Book B) (Ed. R. Goldman, T. Pollard and J. Rosenbaum) p.575, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- COOPER, E.J. TRAUTMANN, M.L. and LASKOWSKI, M. (1950) Occurrence and distribution of an inhibitor for deoxyribonuclease in animal tissues. Proc. Soc. Exp. Biol. Med., 73, 219-222.
- DABROWSKA, W., COOPER, E.J. and LASKOWSKI, M. (1949) A specific inhibitor for deoxyribonuclease. J. Biol. Chem., 177, 991-992.

- ELLIOTT, A., OFFER, G. and BURRIDGE, K. (1976) Electron microscopy of myosin molecules from muscle and non-muscle sources. Proc. R. Soc. Lond. B., 193, 45-53.
- FAIRBANKS, G., THEODORE, L.S. and WALLACH, D.F.H. (1971) Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochem. J., 10, 2606-2617.
- FLOREY, H. (1964) Chemotaxis, phagocytosis and the formation of abscesses. In General Pathology (Third Edition) (Ed. by H. Florey) p.98, Lloyd-Leuke (Medical Books) Ltd., London.
- FLOREY, H.W. and JENNINGS, M.A. (1964) Healing. In General Pathology (Third Edition) (Ed. by H. Florey) p.449, Lloyd-Leuke (Medical Books) Ltd., London.
- FRANKE, W.W., SCHMID, E., OSBORN, M. and WEBER, K. (1978) The intermediate-sized filaments in rat kangaroo PtK<sub>2</sub> cells. II. Structure and composition of isolated filaments. Cytobiologie, 17, 392-411.
- FREDERIC, J. and CHEVREMONT, M. (1952) Recherches sur les chondriosomes des cellules vivantes par la microscopie et la microcinematographie en contraste de phase. Arch. Biol., Paris, 63, 109-131.
- FREED, J.J. and LEBOWITZ, M.M. (1970) The association of a class of saltatory movements with microtubules in cultured cells. J. Cell Biol., 45, 334-354.
- FUJIWARA, K. and POLLARD, T.D. (1976) Fluorescent antibody localization of myosin in the cytoplasm, cleavage furrow and mitotic spindle of human cells. J. Cell Biol., 71, 847-875.

- FUJIWARA, K. and POLLARD, T.D. (1978) Simultaneous localization of myosin and tubulin in human tissue culture cells by double antibody staining. J. Cell Biol., 77, 182-195.
- GABBIANI, G., TRENCH, P. and HOLBOROW, E.J. (1975) Increase of contractile proteins in human cancer cells. LANCET, 2, 796-797.
- GALLAGHER, M., DETWILER, T.C. and STRACHER, A. (1976) Two forms of platelet actin that differ from skeletal muscle actin. In Cell Motility (Book B) (Ed. R. Goldman, T. Pollard and J. Rosenbaum), p.475, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- GERGELY, J. (1976) Troponin-tropomyosin-dependent regulation of muscle contraction by calcium. In Cell Motility (Book A) (Ed. R. Goldman, T. Pollard and J. Rosenbaum), p.137, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- GIBBINS, J.R. (1976) Motility mechanisms in epithelium following injury. J. Cell Biol., 70, 10a.
- GOLDMAN, R.D. (1971) The role of three cytoplasmic fibres in BHK-21 cell motility. I. Microtubules and the effects of colchicine. J. Cell. Biol., 51, 752-762.
- GOLDMAN, R.D. (1972) The effects of cytochalasin B on the microfilaments of baby hamster kidney (BHK-21) cells. J. Cell Biol., 52, 246-254.
- GOLDMAN, R.D. (1975) The use of heavy meromyosin binding as an ultrastructural cytochemical method for localizing and determining the possible functions of actin-like microfilaments in non-muscle cells. J. Histochem. Cytochem., 23, 529-542.



- GOLDMAN, R.D. and KNIPE, D.M. (1973) Functions of cytoplasmic fibres in non-muscle cell motility. Cold Spring Harbor Symp. Quant. Biol., 37, 523-534.
- GOLDMAN, R.D., CHANG, C-M. and WILLIAMS, J. (1975) Properties and behaviour of hamster embryo cells transformed by human adenovirus type 5. Cold Spring Harbor Symp. Quant. Biol., 39, 601-614.
- GOLDMAN, R.D., SCHLOSS, J.A. and STARGER, J.M. (1976) Organizational Changes of Actin-like Microfilaments during Animal Cell Movement. In Cell Motility (Book A) (Ed. by R. Goldman, T. Pollard and J. Rosenbaum), p.217, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- GORDON, D.J., EISENBERG, E. and KORN, E.D. (1976) Characterization of cytoplasmic actin isolated from *Acanthamoeba castellanii* by a new method. J. Biol. Chem., 251, 4778-4786.
- GORDON, III, W.E., BUSHNELL, A. and BURRIDGE, K. (1978) Characterization of the intermediate (10 nm) filaments of cultured cells using an autoimmune rabbit antiserum. Cell, 13, 249-261.
- HANSON, J. and HUXLEY, H.E. (1955) The structural basis of contraction in striated muscle. Symp. Soc. Exp. Biol., 9, 228-264.
- HARTWIG, J.H. and STOSSEL, T.P. (1975) Isolation and properties of actin, myosin and a new actin-binding protein in rabbit alveolar macrophages. J. Biol. Chem., 250, 5699-5705.
- HATANO, S. and OOSAWA, F. (1966) Extraction of an actin-like protein from the plasmodium of a myxomycete and its

- interaction with myosin A from rabbit striated muscle.  
J. Cell. Physiol., 68, 197-202.
- HERMAN, I.M. and POLLARD, T.D. (1978) Actin localization in fixed dividing cells stained with fluorescent heavy meromyosin. Exp. Cell. Res., 114, 15-25.
- HILMEN, M. and SIMON, M. (1976) Motility and the structure of bacterial flagella. Cell Motility (Book A) (Ed. R. Goldman, T. Pollard and J. Rosenbaum), p.35, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- HITCHCOCK, S. (1977) Regulation of motility in non-muscle cells. J. Cell Biol., 74, 1-15.
- HITCHCOCK, S.E., CARLSSON, L. and LINDBERG, U. (1975) Interaction of deoxyribonuclease I with muscle fibrous actin. J. Cell Biol., 67, 172a.
- HITCHCOCK, S.E., CARLSSON, L. and LINDBERG U. (1976) Depolymerization of F-actin by deoxyribonuclease I. Cell, 7, 531-542.
- HUXLEY, H.E. (1963) Electron microscope studies on the structure of natural and synthetic protein filaments from striated muscle. J. Mol. Biol., 7, 281-308.
- HUXLEY, H.E. (1969) The mechanism of muscular contraction. Science, 164, 1356-1366.
- HUXLEY, H.E. (1973) Muscular contraction and cell motility. Nature, 243, 445-449.
- HUXLEY, H.E. (1976) Introductory remarks: the relevance of studies on muscle to problems of cell motility. In Cell Motility (Book A) (Ed. by R. Goldman, T. Pollard and J. Rosenbaum), p.115, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- HUXLEY, H.E. and HANSON, J. (1954) Changes in the cross-striations of muscle during contraction and stretch and their structural interpretation. Nature, 173, 973-976.

- HYNES, R.O. and DESTREE, A.T. (1978) 10 nm filaments in normal and transformed cells. Cell, 13, 151-163.
- ISHIKAWA, H., BISCHOFF, R. and HOLTZER, H. (1968) Mitosis and intermediate-sized filaments in developing skeletal muscle. J. Cell Biol., 38, 538-555.
- ISHIKAWA, H., BISCHOFF, R. and HOLTZER, H. (1969) Formation of arrowhead complexes with heavy meromyosin in a variety of cell types. J. Cell Biol., 43, 312-328.
- KENDRICK-JONES, J., LEHMAN, W. and SZENT-GYÖRGYI, A.G. (1970) Regulation in molluscan muscles. J. Mol. Biol., 54, 313-326.
- KESSLER, D., NACHMIAS, V.T. and LOEWY, A.G. (1971) Actomyosin content of *Physarum* plasmodia and detection of immunological cross-reactions with myosins from related species. J. Cell Biol., 69, 393-406.
- KIRKPATRICK, J.B. (1971) Time-lapse cinematography of axoplasmic flow in peripheral nerves. In Abstracts of the 11<sup>th</sup> Annual Meeting of the American Society for Cell Biology, New Orleans, abstract no. 671.
- KOMNICK, H., STOCKEM, W. and WOHLFARTH-BOTTERMANN, K.E. (1973) Cell Motility: mechanisms in protoplasmic streaming and amoeboid movement. Int. Rev. Cytol., 34, 169-249.
- KORN, E.D. (1978) Biochemistry of actomyosin-dependent cell motility (A review). Proc. Natn. Acad. Sci. U.S.A., 75, 588-599.
- KUNITZ, M. (1950) Crystalline deoxyribonuclease I. Isolation and general properties. Spectrophotometric method for the measurement of deoxyribonuclease activity. J. Gen. Physiol., 33, 349-362.

- KURKI, P., LINDER, E., VIRTANEN, L. and STENMAN, S. (1977) Human auto antibodies reacting with intermediate (100Å) filaments. Nature, 268, 240-241.
- LAEMMLI, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, 680-685.
- LAZARIDES, E. (1976) Actin,  $\alpha$ -actinin and tropomyosin interaction in the structural organization of actin filaments in non-muscle cells. J. Cell Biol., 68, 202-219.
- LAZARIDES, E. and LINDBERG, U. (1974) Actin is the naturally occurring inhibitor of deoxyribonuclease I. Proc. Natn. Acad. Sci. U.S.A., 71, 4742-4746.
- LAZARIDES, E. and WEBER, K. (1974) Actin antibody: The specific visualization of actin filaments in non-muscle cells. Proc. Natn. Acad. Sci. U.S.A., 71, 2268-2272.
- LAZARIDES, E. and HUBBARD, B.D. (1976) Immunological characterization of the subunit of the 100Å filaments from muscle cells. Proc. Natn. Acad. Sci. U.S.A., 73, 4344-4348.
- LAZARIDES, E. and BALZER, Jr., D.R. (1977) Heterogeneity of desmins between mammalian and avian muscles and their coisolation with a specific isoelectric variant of actin. J. Cell Biol., 75, 255a.
- Le BEUX, Y.J. and WILLEMOT, J. (1975) An ultrastructural study of the microfilaments in rat brain by means of heavy meromyosin labelling. Cell Tiss. Res., 160, 1-36.
- Le BEUX, Y.J. and WILLEMOT, J. (1977) Actin-and myosin-like filaments in endothelial cells and pericytes of rat brain capillaries and postcapillary venules. J. Cell Biol., 75, 251a.



- LEHTO, V., VIRTANEN, I. and KURKI, P. (1978) Intermediate filaments anchor the nuclei in nuclear monolayers of cultured human fibroblasts. Nature, 272, 175-177.
- LEWIS, W.H. and LEWIS, M.R. (1924) Behaviour of Cells in Tissue Cultures. In General Cytology (Ed. by S.V. Cowdry), p.385, The University of Chicago Press, Chicago, Illinois, U.S.A.
- LOWEY, A.G. (1952) An actomyosin-like substance from the plasmodium of a myxomycete. J. Cell Comp. Physiol., 40, 127-133.
- LOWEY, S., SLAYTER, H.S., WEEDS, A.G. and BAKER, H. (1969) Substructure of the myosin molecule. J. Mol. Biol., 42, 1-29.
- LOWRY, O.H., ROSENBROUGH, N.J., FARR, L. and RANDALL, R.J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem., 193, 265.
- McMAHAN, U.J. and KUFFLER, S.W. (1971) Visual identification of synaptic boutons on living ganglion cells and of varicosities in postganglionic axons in the heart of the frog. Proc. Royal Soc. B., 177, 485-508.
- MANNHERZ, H.G., BARRINGTON LEIGH, J., LEBERMAN, R. and PERANG, H. (1975) A specific 1:1 G-actin:DNAase I complex formed by the action of DNAase I on F-actin. FEBS Letters, 60, 34-38.
- MATSUMURA, F. and HATANO, S. (1978) Reversible super-precipitation and bundle formation of plasmodium actomyosin. Biochim. Biophys. Acta., 553, 511-523.
- MOORE, P.B., HUXLEY, H.E. and DE ROSIER, D.J. (1970) Three dimensional reconstruction of F-actin, thin filaments and decorated thin filaments. J. Mol. Biol., 50, 279-295.

- MOOSEKER, M.S. and TILNEY, L.G. (1975) Organization of an actin filament-membrane complex. Filament polarity and membrane attachment in microvilli of intestinal epithelial cells. J. Cell Biol., 67, 725-743.
- NACHMIAS, V.T. (1972) Electron microscope observations on myosin from *Physarum polycephalum*. J. Cell Biol., 52, 648-663.
- NIEDERMAN, R. and POLLARD, T.D. (1975) Human platelet myosin II. In vitro assembly and structure of myosin filaments. J. Cell. Biol., 67, 72-92.
- O'FARRELL, P.H. (1975) High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem., 250, 4007-4021.
- OLMSTED, J.B. and BORISY, G.G. (1973) Microtubules. Ann. Rev. Biochem., 42, 507-540.
- OSBORN, M. FRANKE, W. and WEBER, K. (1977) Visualization of a system of filaments 7-10 nm thick in cultured cells of an epithelioid line (PtK2) by immunofluorescence microscopy. Proc. Natn. Acad. Sci. U.S.A., 74, 2490-2494.
- OSTLUND, .R.E., PASTAN, I. and ADELSTEIN, R.S. (1974) Myosin in cultured fibroblasts. J. Biol. Chem., 249, 3903-3907.
- OSTLUND, R.E., Jr., LEUNG, J.T. and KIPNIS, D.M. (1978) Myosins of secretory tissues. J. Cell Biol., 77, 827-836.
- OWARIBE, K. and HATANO, S. (1975) Induction of antibody against actin from myxomycete plasmodium and its properties. Biochemistry, 14, 3024-3029.

- POLLARD, T.D. (1974) Platelet myosin forms three types of paracrystalline tactoids and hybrid filaments with skeletal muscle myosin. J. Cell Biol., 63, 273a.
- POLLARD, T.D. (1976a) Cytoskeletal functions of cytoplasmic contractile proteins. J. Supramol. Struct., 5, 317-334.
- POLLARD, T.D. (1976b) Introductory Remarks: Myosin and Control Mechanisms. In Cell Motility (Book B) (Ed. by R. Goldman, T. Pollard and J. Rosenbaum), p.685, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- POLLARD, T.D. and WEIHING, R.R. (1974) Actin and myosin and cell movement. CRC Crit. Rev. Biochem., 2, 1-65
- POLLARD, T.D. STAFFORD, W.E. and PORTER, M.E. (1978) Characterization of a second myosin from *Acanthamoeba castellanii*. J. Biol. Chem., 253, 4798-4808.
- POLLARD, T.D., FUJIWARA, K., NIEDERMAN, R. and MAUPIN-SZAMIER, P. (1976). Evidence for the role of cytoplasmic actin and myosin in cellular structure and motility. In Cell Motility (Book B) (Ed. by R. Goldman, T. Pollard and J. Rosenbaum), p.689, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- POMERAT, C.M. HENDELMAN, W.J., RAIBORN, Jr., C.W., and MASSEY, J.F. (1967) In The Neuron (Ed. by M. Kyden), p.119, Elsevier, Amsterdam.
- PORTER, K.R. (1976) Introduction: motility in cells. In Cell Motility (Book A) (Ed. by R. Goldman, T. Pollard and J. Rosenbaum), p.1, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- PORTER, K.R., ANDREWS, P. and KELLEY, D. (1972) The preparation of cultured cells and soft tissues for scanning electron microscopy. In: Proc. 5th Annual Stereoscan Colloquium, p.1, Kent Cambridge Scientific, Inc., Chicago.

- RAJU, T.R., STEWART, M. and BUCKLEY, I.K. (1978) Selective extraction of cytoplasmic actin-containing filaments with DNA-ase I. Cytobiologie, 17, 307-311.
- RASH, J.E., McDONALD, T.F., SACHS, H.G. and EBERT, J.D. (1972) Muscle-like arrays in a fibroblast line. Nature New Biol., 237, 160.
- REBHUN, L.I. (1972) Polarized intracellular particle transport: saltatory movements and cytoplasmic streaming. Int. Rev. Cytol., 32, 93-137.
- REES, M.K. and YOUNG, M. (1967) Studies on the isolation and molecular properties of homogeneous globular actin. J. Biol. Chem., 242, 4449-4458.
- RICE, R.V. and BRADY, A.C. (1973) Biochemical and ultrastructural studies on vertebrate smooth muscle. Cold Spring Harbor Symp. Quant. Biol., 37, 429-438.
- ROSE, G.G., POMERAT, C.M., SHINDLER, T.O. and TRUNNELL, J.B. (1958) A cellophanestrip technique for culturing tissue in multipurpose culture chambers. J. Biophys. Biochem. Cytol., 4, 761-764.
- SANGER, J.W. (1975) Changing patterns of actin localization during cell division. Proc. Natn. Acad. Sci. U.S.A., 72, 1913-1916.
- SCHOLLMAYER, J.E., FURCHT, L.T., GOLL, D.E., ROBSON, R.M. and STROMER, M.H. (1976) Localization of contractile proteins in smooth muscle cells and in normal and transformed fibroblasts. In Cell Motility (Book A) (Ed. by R. Goldman, T. Pollard and J. Rosenbaum), p.361, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- SHEETZ, M.P., PAINTER, R.C. and SINGER, S.J. (1976) Relationships of the spectrin complex of human erythrocyte membranes to the actomyosins of muscle cells. Biochemistry, 15, 4486-4491.



- SHELANSKI, M.L., YEN, S-H. and LEE, V.M. (1976)  
Neurofilaments and glial filaments. In Cell Motility  
(Book C) (Ed. by R. Goldman, T. Pollard and J. Rosenbaum),  
p. 1007, Cold Spring Harbor Laboratory, Cold Spring  
Harbor, New York.
- SHOENBERG, C.F. and NEEDHAM, D.M. (1976) A study of the  
mechanism of contraction in vertebrate smooth muscle.  
Biol. Rev., 51, 53-104.
- SLAYTER, H.S. and LOWEY, S. (1967) Substructure of the  
myosin molecule as visualized by electron microscopy.  
Proc. Natn. Acad. Sci. U.S.A., 58, 1611-1618.
- SMALL, J.V. and SQUIRE, J.M. (1972) Structural basis of  
contraction in vertebrate smooth muscle. J. Mol. Biol.,  
67, 117-149.
- SMALL, J.V. and SOBIESZEK, A. (1977) Studies on the function  
and composition of the 10 nm (100Å) filaments of  
vertebrate smooth muscle. J. Cell Sci., 23, 243-268.
- SMALL, J.V., ISENBERG, G. and CELIS, J.E. (1978) Polarity  
of actin of cultured cells. Nature, 272, 638-639.
- SPOONER, B.S., YAMADA, K.M. and WESSELLS, N.K. (1971)  
Microfilaments and cell locomotion. J. Cell Biol., 49,  
595-613.
- SPOONER, B.S., ASH, J.F., WRENN, J.T., FRATER, R.B. and  
WESSELLS, N.K. (1973) Heavy meromyosin binding to  
microfilaments involved in cell and morphogenetic  
movements. Tissue Cell, 5, 37-46.
- SPUDICH, J.A. and WATT, S. (1971) The regulation of rabbit  
skeletal muscle contraction. J. Biol. Chem., 246,  
4866-4871.

- STARGER, J.M. and GOLDMAN, R.D. (1977) Isolation and preliminary characterization of 10 nm filaments from baby hamster kidney (BHK-21) cells. Proc. Natn. Acad. Sci. U.S.A., 74, 2422-2426.
- STARGER, J.M., BROWN, W.E., GOLDMAN, A.E. and GOLDMAN, R.D. (1978) Biochemical and immunological analysis of rapidly purified 10 nm filaments from baby hamster kidney (BHK-21) cells. J. Cell Biol., 78, 93-109.
- STORTI, R.V. and RICH, A. (1976) Chick cytoplasmic actin and muscle actin have different structural genes. Proc. Natn. Acad. Sci. U.S.A., 73, 2346-2350.
- STOSSEL, T.P. and HARTWIG, J.H. (1975) Interactions between actin, myosin and an actin-binding protein from rabbit alveolar macrophages. J. Biol. Chem., 250, 5706-5712.
- STOSSEL, T.P. and HARTWIG, J.H. (1976) Phagocytosis and the contractile proteins of pulmonary macrophages. In Cell Motility (Book B) (Ed. by R. Goldman, T. Pollard and J. Rosenbaum), p.529, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
- SUMMERS, K.E. and GIBBONS, I.R. (1971) Adenosine triphosphate - induced sliding of tubules in trypsin-treated flagella of sea-urchin sperm. Proc. Natn. Acad. Sci. U.S.A., 68, 3092-3096.
- SZENT-GYÖRGYI, A. (1947) In Chemistry of muscular contraction. Academic Press Inc., New York.
- SZENT-GYÖRGYI, A.G. (1953) Meromyosins, the subunits of myosin. Arch. Biochem. and Biophys., 42, 305-320.
- TILNEY, L.G. (1975) Actin filaments in the acrosomal reaction of *Limulus* sperm. J. Cell Biol., 64, 289-310.

- TILNEY, L.G. (1977) Actin: its association with membranes and the regulation of its polymerization. In International Cell Biology (Ed. by B.R. Brinkley and K.R. Porter), p.388, The Rockefeller University Press, New York.
- WEBER, K. and GROESCHEL-STEWART, U. (1974) Antibody to myosin: the specific visualization of myosin-containing filaments in non-muscle cells. Proc. Natn. Acad. Sci. U.S.A., 71, 4561-4564.
- WESSELS, N.K., SPOONER, B.S. and LUDUEÑA, M.A. (1973) Surface movements, microfilaments and cell locomotion. In Locomotion of Tissue Cells (New Series) (Ed. by R. Porter and D.W. Fitzsimons), p.53, CIBA Foundation Symposium, 14 Elsevier, Amsterdam, London and New York.
- WILLIAMSON, R.E. (1974) Actin in the alga *Chara corallina*. Nature, 248, 801-802.
- WILLIAMSON, R.E. (1975) Cytoplasmic streaming in *Chara*: a cell model activated by ATP and inhibited by cytochalasin B. J. Cell Sci., 17, 655-668.
- WUERKER, R.B. (1970) Neurofilaments and glial filaments. Tissue Cell, 2, 1-9.
- YAGI, K., MASE, R., SAKAKIBARA, I. and ASAI, H. (1965) Function of heavy meromyosin in the acceleration of actin polymerization. J. Biol. Chem., 240, 2448-2454.
- YAMADA, K.M., SPOONER, B.S. and WESSELLS, N.K. (1971) Ultrastructure and function of growth cones and axons of cultured nerve cells. J. Cell Biol., 49, 614-635.
- YANG, Y-Z. and PERDUE, J.F. (1972) Contractile proteins of cultured cells. I. The isolation and characterization of an actin-like protein from cultured chick embryo fibroblasts. J. Biol. Chem., 247, 4503-4509.

ZUCKER-FRANKLIN, D. and GRUSKY, G. (1972) The actin  
and myosin filaments of human and bovine blood platelets.  
J. Clin. Invest., 51, 419-430.